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BRITISH JOURNAL OF CANCER

VOL. XV MARCH, 1961 NO. 1

CANCER MORTALITY TRENDS IN CANADA—1941 TO 1958

A. J. PHILLIPS

From the National Cancer Institute of Canada, 790 Bay St., Toronto, Canada

Received for publication January 23, 1961

THIS paper presents an analysis by sexes of the deaths in Canada assigned to nine of the major sites of cancer and to leukaemia. The sites which have been selected for study with their respective International List (Detailed) numbers are:

Buccal cavity	140–148	Urinary organs	180–181
Stomach	151	Breast (female)	170
Intestines	152–153	Uterus	171–174
Rectum	154	Genital organs (male)	177–179
Respiratory system	161–163	Leukaemia	204
		All sites	140–205

The period under review covers the years 1941 to 1958 inclusive. The year 1941 was selected as a suitable starting point because in that year, such sites of cancer as intestine and stomach began to be listed separately in the annual Vital Statistics reports for Canada.

The statistical procedures which have been used in the analysis of the data may be explained briefly as follows. First, the mortality rates for each site and sex were age-adjusted to the Canada 1951 census population as a standard. This procedure accounts for changes in rates which are due to changes in the age-structure of a population in the course of time. Since cancer death rates increase greatly with age and since the population of Canada is aging—that is, the relative number of older individuals is increasing, it is essential that this age-adjustment be carried out whenever any comparison of rates in time is made.

Second, the age-adjusted rates for the period 1941–1949 were made comparable with the years 1950–1958 by means of comparability ratios calculated by the Dominion Bureau of Statistics on the deaths in Canada in 1949. In that year the sixth revision of the International Classification of Diseases, Injuries and Causes of Death was published and, in order to assess the variations between this revision and the previous one, all deaths in Canada in 1949 were tabulated by both. From this dual tabulation the comparability ratios were calculated.

Third, the trend line for each set of age-adjusted rates was found. This line is, among all possible lines, the one for which the sum of the squared vertical distances from it to the points representing the rates is a minimum. It is commonly known as the "best-fitting" line. If this line tends to rise through the time interval under consideration (1941–1958) it has a positive slope. If the line falls the slope is negative and if it is horizontal the slope is zero. When the line has a positive or

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negative slope it signifies that the rates have increased or decreased during the interval and the probability is computed that a true rise or drop has occurred. This probability depends upon two factors : the magnitude of the slope of the line, and the amount of scatter of the actual points about the line. The probability value of less than 0·05 was considered sufficiently small to call a slope significantly different from zero. Hence, a "significant " trend indicates that the probability of obtaining, through chance alone, a set of rates which would give rise to this trend, or one at least as pronounced, is less than 5 in 100. It will be noted that the rates for some sites are increasing or decreasing but the subsequent tests of significance show that the increase or decrease may be due to chance variation. This illustrates the weakness of interpreting mortality trends from an inspection of death rates and points out the necessity for testing for significance.

The following example of the fitting of the trend line is based upon the age-adjusted death rates for cancer of the stomach in males.

Example : based upon age-adjusted death rates for cancer of the stomach in males.

Year	Death rate (Y)	Time (X)	Y'	$(Y - Y')$
1941	31·7	−8·5	31·4	0·3
1942	30·9	−7·5	31·0	−0·1
1943	30·9	−6·5	30·5	0·4
1944	29·9	−5·5	30·1	−0·2
1945	30·2	−4·5	29·6	0·6
1946	28·2	−3·5	29·2	−1·0
1947	29·0	−2·5	28·8	0·2
1948	27·9	−1·5	28·3	−0·4
1949	27·5	−0·5	27·9	−0·4
1950	26·6	0·5	27·4	−0·8
1951	27·2	1·5	27·0	0·2
1952	28·0	2·5	26·6	1·4
1953	25·4	3·5	26·1	−0·7
1954	25·6	4·5	25·7	−0·1
1955	25·7	5·5	25·3	0·4
1956	25·0	6·5	24·8	0·2
1957	24·3	7·5	24·4	−0·1
1958	23·9	8·5	24·0	−0·1

ΣY = sum of $Y = 497 \cdot 9$.
$\overline{Y}$ = mean of $Y = 27 \cdot 66$.
ΣXY = sum of products of X and $Y = -211 \cdot 8$.
ΣX^2 = sum of squares of $X = 484 \cdot 5$.

$$\text{SLOPE } (m) = \frac{\Sigma XY}{\Sigma X^2} = -0 \cdot 437$$

$$Y' = mX + \overline{Y}$$

$$\Sigma(Y - Y')^2 = 5 \cdot 38$$

$$S_m = \text{standard deviation of slope} = \sqrt{\frac{\Sigma(Y - Y')^2}{(n - 2)\ (\Sigma X^2)}} = \sqrt{\frac{5 \cdot 38}{16 \times 484 \cdot 5}} = 0 \cdot 026$$

$$t = \text{Student's " t " test} = \frac{|\ m\ |}{S_m} = \frac{0 \cdot 437}{0 \cdot 026} = 16 \cdot 8$$

The number of degrees of freedom is 16 when $n = 18$ and from the Table of " t " the probability that this trend is due to chance is less than 0·001 or one in one thousand.

It is concluded, therefore, that there has been a significant drop in the age-adjusted death rate for cancer of the stomach in Canadian males during this interval of time.

Analyses of specific sites of cancer

The age-adjusted mortality rates for the nine sites of cancer, for leukaemia, and for all sites combined are shown for males in Table I and for females in Table II. The trend line has been fitted to each set of rates and the appropriate test of significance made. The results may be summarized as follows:

TABLE I.—*Age-adjusted Mortality Rates Per* 100,000 *Population in Canada for Various Sites of Cancer—Male*

Year	Buccal cavity	Stomach	Intestines	Rectum	Respiratory system	Urinary organs	Genital system	Leukaemia	All sites
1941	7·2	31·7	15·0	7·1	9·1	7·4	12·9	3·8	123·2
1942	7·0	30·9	15·2	7·4	8·9	7·6	12·4	3·6	121·9
1943	7·1	30·9	15·6	7·5	9·9	7·8	12·6	3·9	124·7
1944	6·5	29·9	14·8	8·5	9·8	7·7	12·1	4·2	122·7
1945	6·1	30·2	15·6	7·8	10·4	7·7	11·9	4·0	123·5
1946	5·7	28·2	14·9	7·5	11·9	8·0	12·5	4·3	122·9
1947	5·9	29·0	15·2	7·9	13·3	8·1	13·5	4·8	128·9
1948	6·1	27·9	14·9	8·3	13·9	8·5	13·3	4·7	129·7
1949	5·9	27·5	14·9	7·8	15·6	8·1	14·1	4·9	131·0
1950	5·4	26·6	14·6	8·2	16·5	8·4	14·1	4·8	128·5
1951	4·9	27·2	14·0	7·0	17·3	8·4	13·4	5·2	131·4
1952	4·9	28·0	16·0	7·2	19·2	8·2	14·4	5·3	136·8
1953	4·7	25·4	14·9	8·0	20·4	8·3	14·5	6·3	136·4
1954	4·8	25·6	14·3	8·4	21·6	8·8	13·9	6·2	137·6
1955	5·2	25·7	14·5	8·8	22·9	9·4	14·1	6·1	141·0
1956	5·2	25·0	14·9	8·2	24·7	9·8	14·5	6·3	142·4
1957	4·7	24·3	15·2	8·4	25·4	9·1	13·8	6·4	142·5
1958	4·3	24·9	15·1	8·1	26·3	8·8	14·1	6·8	143·4

TABLE II.—*Age-adjusted Mortality Rates Per* 100,000 *Population in Canada for Various Sites of Cancer—Female*

Year	Buccal cavity	Stomach	Intestines	Rectum	Respiratory system	Urinary organs	Breast	Cervix uteri	Corpus uteri	Leukaemia	All sites
1941	1·4	18·1	17·8	5·1	3·2	3·9	22·7	7·9	9·7	2·9	124·6
1942	1·7	17·9	18·1	5·1	3·1	4·2	22·5	7·1	11·3	3·0	124·6
1943	1·6	17·9	18·3	5·4	3·6	4·0	23·4	6·5	10·8	2·6	125·4
1944	1·4	16·8	19·8	5·3	3·9	4·3	22·1	7·0	10·4	3·4	124·9
1945	1·3	15·3	19·0	5·1	3·4	4·1	22·7	8·0	9·9	3·0	122·8
1946	1·2	16·8	18·9	5·2	3·4	3·9	23·3	6·9	10·2	3·5	123·8
1947	1·3	14·9	18·4	5·5	4·8	4·3	22·4	7·8	9·6	3·3	124·0
1948	1·3	16·1	18·7	5·5	3·7	4·1	23·5	7·6	9·0	3·4	126·1
1949	1·3	14·6	17·2	5·1	4·4	4·4	22·7	7·9	8·3	4·3	122·9
1950	1·2	14·3	18·0	4·8	3·3	4·6	22·9	8·0	7·3	4·5	121·0
1951	1·3	13·8	18·5	5·6	3·7	4·1	22·9	9·0	7·4	3·9	123·3
1952	1·3	14·0	17·8	5·2	4·0	4·0	23·2	8·7	6·7	3·9	121·0
1953	1·4	14·1	17·3	5·0	3·6	3·9	24·1	8·1	5·9	4·1	122·0
1954	1·4	13·6	16·8	4·8	3·8	4·0	23·2	7·9	6·8	3·5	120·9
1955	1·3	12·6	17·4	4·6	4·6	4·2	22·6	7·8	5·4	4·7	119·6
1956	1·4	12·6	16·6	5·1	4·1	3·9	22·8	8·3	5·7	4·3	119·6
1957	1·3	11·6	16·4	5·1	3·9	3·9	23·6	8·0	5·5	4·5	120·2
1958	1·2	11·4	17·0	5·1	3·8	4·0	23·2	7·6	5·6	4·7	118·5

Buccal cavity.—This analysis includes deaths assigned to cancer of the lip, tongue, salivary gland, floor of mouth, other parts of mouth, oral mesopharynx, nasopharynx, hypopharynx and pharynx (unspecified). The slopes of the trend lines show that there has been a significant decrease in mortality for males (38·6 per cent) but no change for females (Fig. 1).

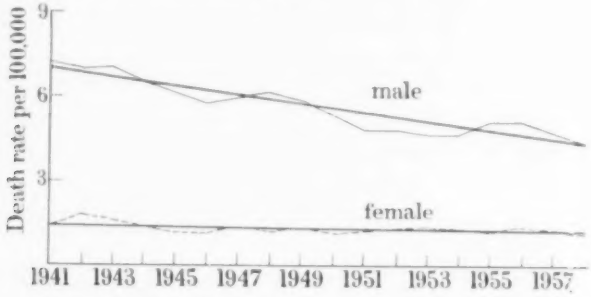


FIG. 1.—Mortality trends in Canada—buccal cavity.
Male—significant. Female—not significant.

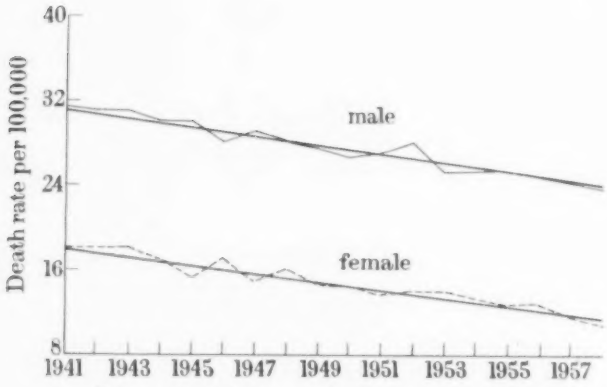


FIG. 2.—Mortality trends in Canada—stomach.
Male—significant. Female—significant.

Stomach.—The slopes of the trend lines for mortality from cancer of the stomach reflect significant declines for males and for females. Over the period 1941–1958 the male death rate has dropped 23·6 per cent and the female 35·5 per cent (Fig. 2).

Intestines.—It will be noted that the age-adjusted mortality rates for females are consistently higher than those for males throughout the period. The slope of the trend line for males shows no significant change but that for females shows a significant decrease (11·6 per cent) in the age-adjusted rates (Fig. 3).

Rectum.—The analysis of the deaths ascribed to cancer of the rectum indicates no significant change in either male or female mortality (Fig. 4).

Respiratory system.—This analysis includes deaths attributed to cancer of the trachea, bronchus and lung not specified as secondary. There has been a significant increase in male mortality but in females, although the trend line indicates an increase of 20 per cent between 1941 and 1958, the probability that this might be due to chance is about 0·07. In males the age-adjusted death rate has increased

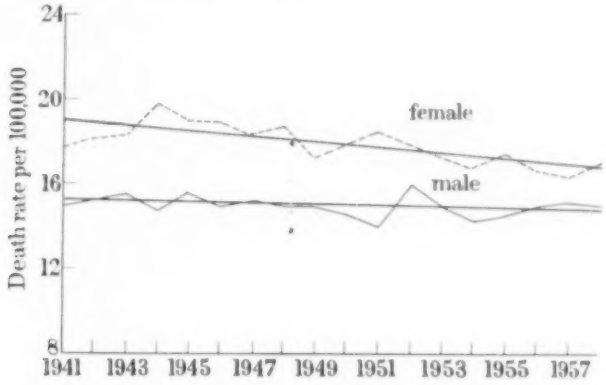


FIG. 3.—Mortality trends in Canada—intestine.
Male—not significant. Female—significant.

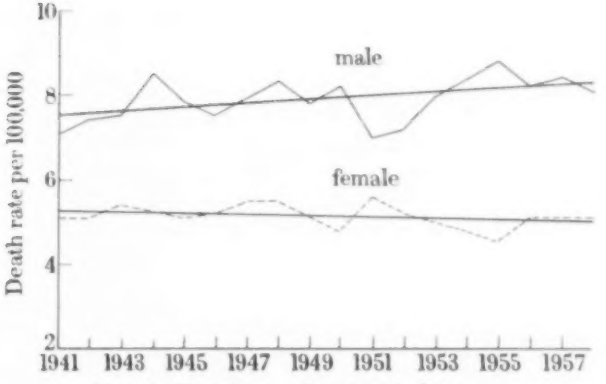


FIG. 4.—Mortality trends in Canada—rectum.
Male—not significant. Female—Not significant.

271·4 per cent over the years 1941–1958 according to the fitted trend line, but as the graph indicates a single straight line does not produce a satisfactory fit throughout the whole period, the rate of increase being apparently greater after 1945 than it was during 1941–1945. (Fig. 5.)

Urinary organs.—The slopes of the trend lines indicate no significant change in the female rates. The male rate increased 24·3 per cent (Fig. 6).

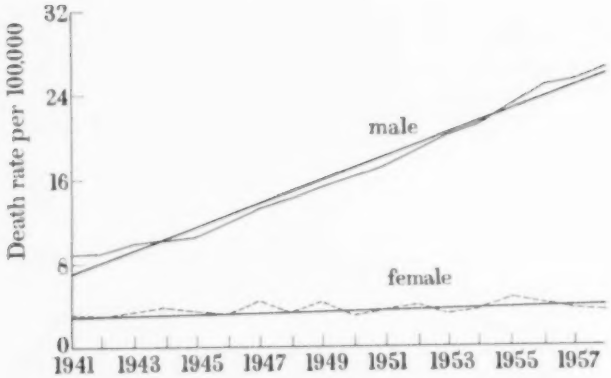


FIG. 5.—Mortality trends in Canada—respiratory system.
Male—significant. Female—not significant.

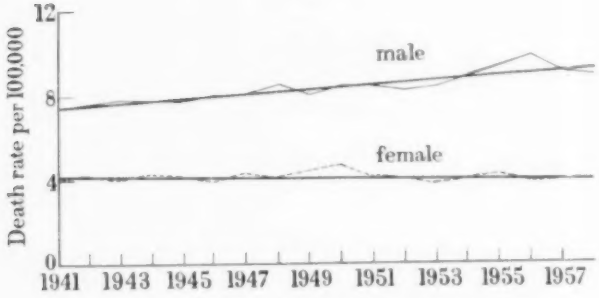


FIG. 6.—Mortality trends in Canada—urinary organs.
Male—significant. Female—not significant.

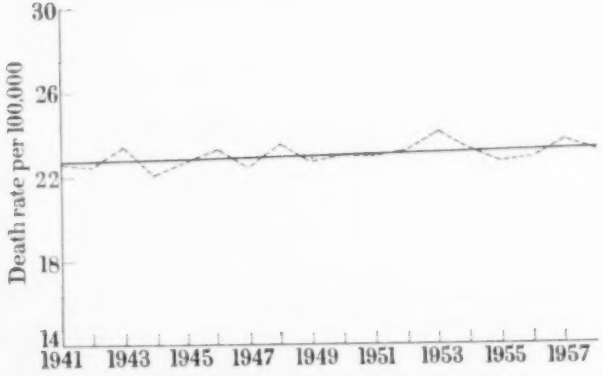


FIG. 7.—Mortality trends in Canada—breast.
Female—not significant.

Breast.—The analysis of the deaths attributed to breast cancer in females indicates that no significant change in mortality occurred in the period 1941–1958 (Fig. 7).

Uterus.—Age-adjusted mortality rates have been calculated separately for deaths assigned to cancer of the cervix uteri and those assigned to the corpus

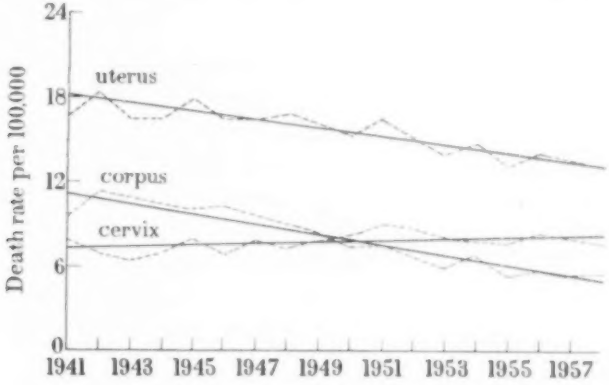


FIG. 8.—Mortality trends in Canada—uterus.
Uterus—significant.
Corpus—significant.
Cervix—significant.

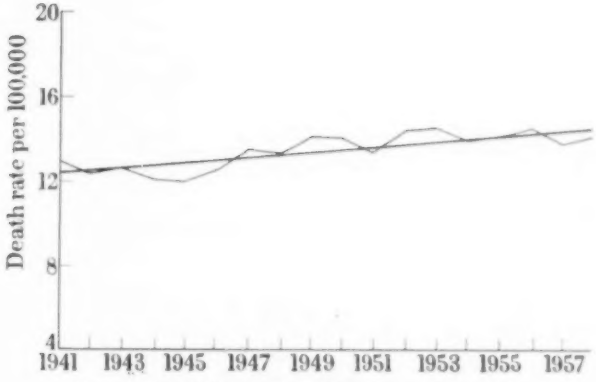


FIG. 9.—Mortality trends in Canada—male genital.
Male—significant.

uteri. The trend line for cancer of the cervix shows an increase in the rates of 13·7 per cent which is of borderline significance : the probability that the trend is due to chance lies between the 0·05 and the 0·02 levels of confidence. The slope of the trend line for the corpus uteri indicates a significant decrease (55·4 per cent) in the mortality rates. The slope of the trend line for the uterus including the cervix indicates a significant decrease of 28·7 per cent in the age-adjusted rates (Fig. 8).

Male genital organs.—This analysis includes deaths attributed to cancer of the prostate, testis and other male genital organs. The slope of the trend line indicates that a significant increase in mortality rates (16·9 per cent) occurred over the period being studied. (Fig. 9.)

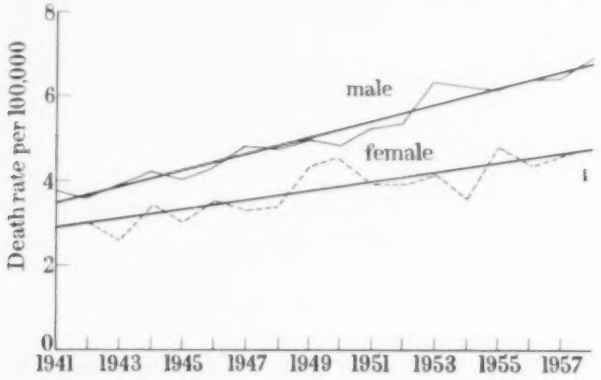


Fig. 10.—Mortality trends in Canada—leukaemia.
Male—significant. Female—significant.

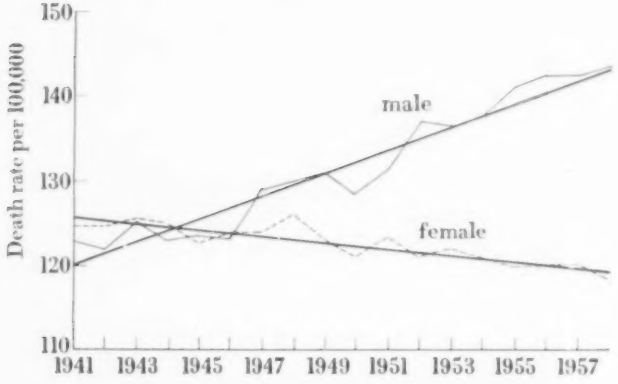


Fig. 11.—Mortality trends in Canada—all sites.
Male—significant. Female—significant.

Leukaemia.—The analysis shows a significant increase in the mortality rates for males and females. The male rates increased 91·4 per cent and the female rates 62·1 per cent (Fig. 10).

All sites of cancer.—The analysis of the deaths attributed to cancer of all sites, including leukaemia and the lymphatic and haemotopoietic system, shows that a significant increase has occurred in male mortality and a significant decrease in female mortality (Fig. 11).

SUMMARY

An analysis has been made of the recorded deaths in Canada attributed to cancer during the period 1941–1958. The trends in the age-adjusted death rates for each site and sex have been tested for significant increases or decreases by means of Students' "*t*" test.

Significant decreases in mortality were found in

(*a*) cancer of the buccal cavity . . . . . male
(*b*) cancer of the stomach . . . . . male
(*c*) cancer of the stomach . . . . . female
(d) cancer of the intestine . . . . . female
(e) cancer of the corpus uteri . . . . .
(f) cancer of all sites . . . . . female

Significant increases in mortality were found in

(a) cancer of the respiratory system . . . . male
(b) cancer of the urinary organs . . . . male
(c) cancer of the cervix uteri . . . . .
(d) cancer of the genital organs . . . . . male
(e) leukaemia . . . . . male
(f) leukaemia . . . . . female
(g) cancer of all sites . . . . . male

No significant changes in mortality were found in

(a) cancer of the buccal cavity . . . . . female
(b) cancer of the intestine . . . . . male
(c) cancer of the rectum . . . . . male
(d) cancer of the rectum . . . . . female
(e) cancer of the respiratory system . . . . female
(f) cancer of the urinary organs . . . . female
(g) cancer of the breast . . . . . female

The assistance of the Dominion Bureau of Statistics is gratefully acknowledged.

THE RELATIONSHIP BETWEEN CANCER AND TUBERCULOSIS MORTALITY RATES

A. H. CAMPBELL

From the Repatriation Department, Victoria, Australia

Received for publication January 3, 1961

ATTENTION was first drawn to the peculiar relationship between the tuberculosis and cancer mortality rates by the New South Wales Government Statistician (Coghlan, 1902). He demonstrated that in certain age groups the joint death rates due to tuberculosis and cancer remained relatively constant. As the death rate from tuberculosis decreased, it was almost exactly balanced by an increase of the death rate from malignancy. In the succeeding years, the joint death rates have not remained constant within age groups.

However, the phenomenon has been extensively investigated by Cherry (1924, 1925, 1933, 1935, 1936, 1940, 1945). He found that in England the joint death rate from cancer and phthisis (pulmonary tuberculosis) had remained constant for many years. Moreover, he found a similar relationship when the mortality rates were arranged to obtain information concerning groups of people identified by the year or decade of birth. Such groups are termed generation units, cohorts, or census units. The total deaths for each unit represents the total number of individuals in that generation. For a period of four census units, from the age 15 to 75 years, the ratio of

$$\frac{\text{deaths from phthisis and cancer}}{\text{deaths from all causes}}$$

remained practically constant, both for males and females. The total deaths from cancer and phthisis constantly caused approximately 20 per cent of the total deaths of each generation unit.

Cherry examined the death rates from other conditions and could find no other two conditions with a joint mortality rate approaching a constant. The relationship between the phthisis and cancer mortality appeared to be unique.

Cruikshank (1939) confirmed some of Cherry's observations and extended them by showing that the relationship applied not only to phthisis and cancer but also to tuberculosis, including extra-pulmonary forms, and cancer.

The constancy of the ratio in Table I (from Cruikshank) means that over the period covered, the decrease in deaths from phthisis or tuberculosis has been exactly compensated by an increase in deaths from cancer.

Cruikshank also established that within each generation unit, the distribution of deaths from tuberculosis and cancer follows a specific pattern according to age. If the logarithm of the ratio of $\frac{\text{Cancer deaths}}{\text{Tuberculosis deaths}}$ (for each age group over 25 years) is plotted against the corresponding age groups, a graph is obtained which is a straight line.

TABLE I.—20 *Per Cent Ratios for Males*

Census unit	Deaths phthisis and cancer / Deaths all causes.		Deaths Tb and Cancer / Deaths all causes.	
	Uncorrected	corrected*	Uncorrected	corrected*
1	21·00	22·09	21·70	23·16
2	21·54	22·40	22·21	23·15
3	22·14	22·36	22·83	23·14
4	22·61	22·61	23·34	23·42
Mean	21·82	22·37	22·52	23·22
S.D.	0·61	0·19	0·62	0·12
Coef. var.	2·79	0·83	2·76	0·5

* Corrected for improvements in diagnosis etc.

Where $R = \frac{\text{deaths Cancer,}}{\text{deaths Tb.}}$ values of R increase by a definite law which takes the form $R_t = R_0 e^{kt}$.

This elegant mathematical relationship of deaths from two diseases affecting each generation unit, adds to the possibility that the relationship is more than fortuitous.

Paxon (1956) confirmed that the joint death rates from tuberculosis and cancer had remained relatively constant in England and Wales up to 1950. He added the observation that for males, and to a lesser extent for females, the fall in deaths from non-pulmonary tuberculosis has been balanced by the increased number of deaths from pulmonary cancer. Similarly, the total deaths from extra-pulmonary malignancy and the deaths from pulmonary tuberculosis have balanced each other.

Cherry, Cruikshank and Paxon regarded the relationships between the mortality rates as evidence of a causal relationship between tuberculosis and cancer. However, Pearl (1929) has criticised conclusions drawn from the crude mortality rates and correctly pointed out that these rates are influenced by age changes in the population.

Consequently, any explanation of the constancy of the joint death rates must make allowance for the influence of ageing of the population. Therefore, a simple causal connection between tuberculosis and cancer is necessarily an inadequate explanation of this and of the other associations.

Cherry has made interesting observations that in many occupational groups, localities or countries, the phthisis and cancer rates vary in a parallel manner. A high phthisis rate is accompanied by a high cancer rate and a low tuberculosis rate by a low cancer rate. This finding is in contrast to the behaviour of the mortality rates, over a period of time, when decrease of the phthisis rate is accompanied by a rise and not a fall in the cancer rate.

Cherry (1940) was able to show that in groups of people following the same occupation or living in the same area, the mortality from tuberculosis and cancer usually ran parallel.

" In Melbourne the population of one million was divided into three sections by taking the suburbs with the highest, lowest, and intermediate rates for cancer. In the five years period, 1931–35, the highest had an excess of 58 per cent in males and 32 per cent in females, the population of these two sections being approximately equal. There was a similar distribution of the deaths from tuberculosis, the excess n the highest cancer suburbs being 38 per cent for males and 24 per cent for females.

The highest rates were found in the most densely peopled industrial suburbs, and the lowest in the semi-rural suburban areas."

Pearl (1929) employed age-specific mortality rates in place of the crude mortality rates, for 22 American States. He confirmed that tuberculosis and cancer mortality varied in a like manner when localities were compared. In various age groups, there was a close correlation between the tuberculosis mortality rate and, ten years later, the cancer mortality rate in the age group ten years older.

INVESTIGATION OF AUSTRALIAN MORTALITY RATES

The purpose of this investigation has been to determine whether the Australian mortality rates from tuberculosis and malignant neoplasms have the same relationships as those reported by Cherry, Cruikshank, and Paxon for England and Wales.

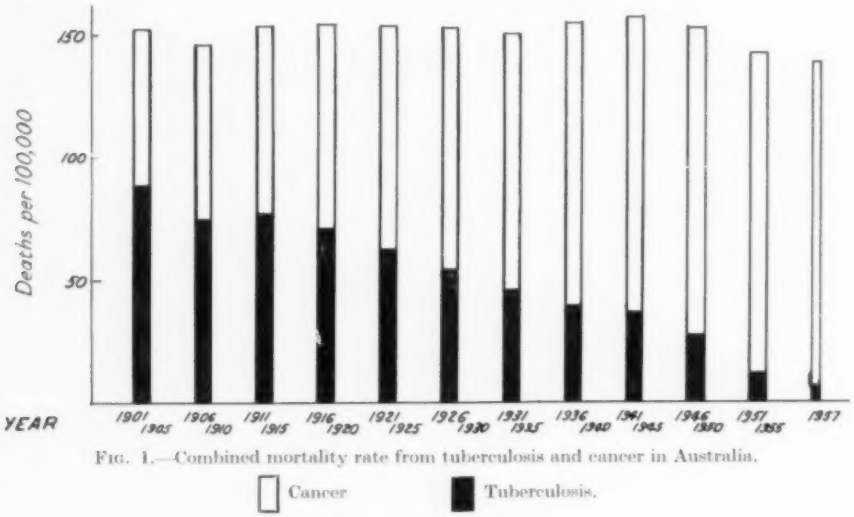


FIG. 1.—Combined mortality rate from tuberculosis and cancer in Australia.

RESULTS

Joint death rates—tuberculosis and cancer

The Australian mortality rates from tuberculosis and malignant neoplasms (all types) from 1900 until 1957 have been plotted in Figs. 1 and 2.

It is apparent that the combined crude mortality rate from tuberculosis and cancer has remained remarkably constant for fifty years. This is true for the population as a whole (Fig. 1) and for the male and female population separately (Fig. 2). The graphs show a slight fall in the joint rate within recent years and some slight fluctuation during the two War periods.

The separate Queensland mortality rates have been charted in Fig. 3. Once again, the relative constancy of the combined mortality rate of tuberculosis and cancer is a striking feature. In both Australia and Queensland the decrease in

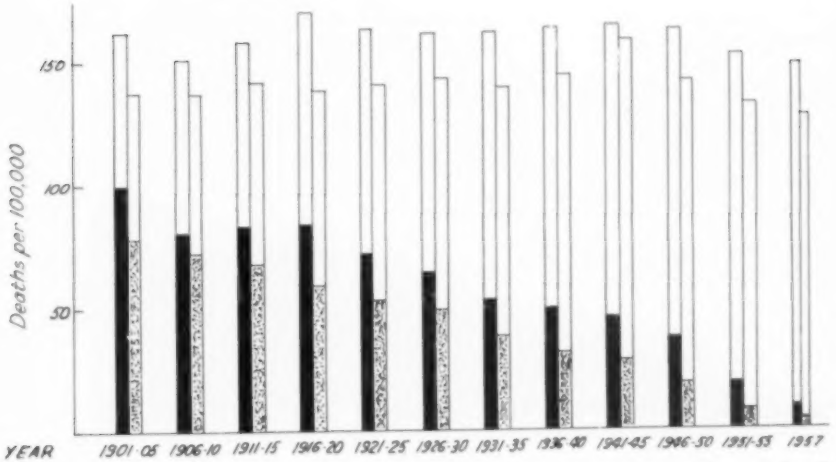
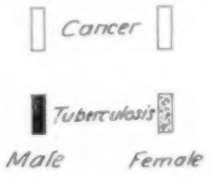


FIG. 2.—Combined mortality rate from tuberculosis and cancer in Australia. Males and females.



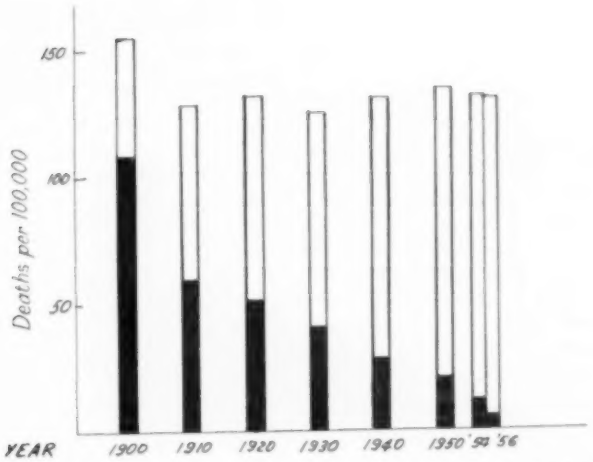


FIG. 3.—Combined mortality rate from tuberculosis and cancer in Queensland.

mortality from tuberculosis has been matched by an increase of crude mortality from cancer.

Difference between the mortality rates in different localities

Although the joint mortality rates remain relatively constant in each locality, the combined rates and the separate rates for tuberculosis and cancer differ according to locality.

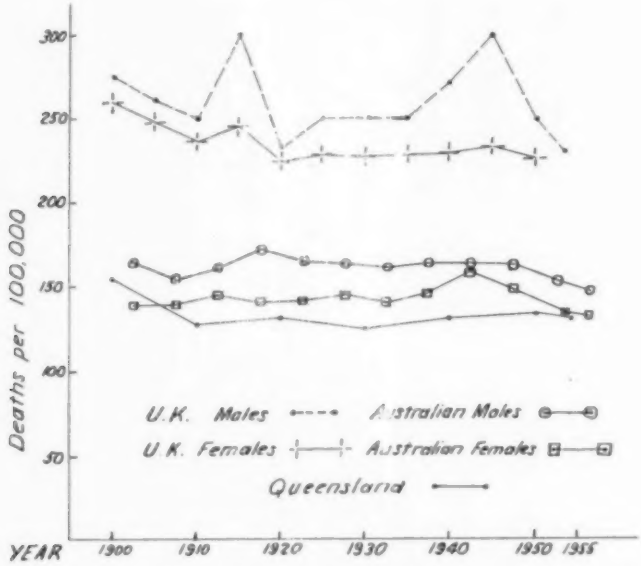


FIG. 4.—Comparison of combined mortality rates, tuberculosis and cancer in various regions.

The combined mortality rates for tuberculosis and cancer have been plotted in Fig. 4 for English males and females, Australian males and females, and the Queensland population. This clearly demonstrates the constancy of the combined rates according to locality and the vast differences between localities. The combined death rate from cancer and tuberculosis in Queensland is almost half that of the males in England. The differences between localities have remained almost constant during the last fifty years.

When locality mortalities are compared with each other, tuberculosis and cancer death rates vary in a parallel manner. A high tuberculosis rate is accompanied by high cancer rate. Comparisons are made of the average mortality rates for England and Wales (1926–1935), Australia (1926–1935), and Queensland (1930). The parallel relationship between tuberculosis and cancer found when localities are compared (Table II) is in direct contrast with the temporal changes within each locality, where a fall in tuberculosis rate is matched by a rise in the cancer rate.

TABLE II.—*Comparison of Death Rates in Different Localities* (*per* 100,000)

Cause of death	Queensland	Australia	England
Tuberculosis . .	42	51	89
Cancer . . .	82	100	146
Total . . .	124	151	235

DISCUSSION

In the past, the relationship between the total cancer mortality and the tuberculosis mortality rates has been quoted as evidence of a causal relationship between tuberculosis and all types of neoplasms.

Coghlan (1902) postulated that a common diathesis may render people susceptible to either tuberculosis or cancer. A reduction of tuberculosis mortality would then lead to an increase of cancer mortality. There is no direct evidence to support this theory. When occupations and social classes are examined, it is found that a high rate for tuberculosis is accompanied by a high cancer rate, and a low tuberculosis rate by a low cancer rate. Therefore, area or occupation groups with a low tuberculosis rate fail to show evidence of a "salvage" population which develops malignancy.

As tuberculosis and cancer mortality were commonly high in the same occupational and locality groups, Cherry believed that tuberculosis was the specific precursor of cancer. Paxon (1956) has followed Cherry in postulating a positive relationship especially for carcinoma of the lung.

Cruikshank (1939) has elaborated Cherry's theory and put forward the hypothesis that the tubercle bacillus carries a carcinogenic phage. He postulates that infection with the tubercle bacillus may lead to the development of tuberculosis or if the bacilli do not flourish, the phage becomes liberated to cause cancer. This theory is illustrated by considerable mathematical argument.

These authors accept as evidence of a positive relationship two sets of facts which seem at first sight to be contradictory. In the first place, a high tuberculosis rate associated with a high cancer rate in a locality, a country, or an occupation is suggestive of a possible causal relationship. Within the same country, locality or occupation if a high tuberculosis rate is reduced (in time) then this is accompanied by more, not less, cancer at all ages. These opposing associations are unlikely to arise from a common cause. For example, in comparison with Australia and New Zealand, a high tuberculosis mortality in Britain is associated with a high cancer mortality. If this is due to a causal relationship, lowering of the tuberculosis mortality in Britain should be associated with a lowering of the cancer mortality rate instead of the increased rate that has occurred.

The exact opposite hypothesis that tuberculosis inhibits the development of cancer (Pearl, 1929) would explain why cancer increases as tuberculosis decreases. However, this theory does not explain why tuberculosis and cancer flourish in certain areas and living conditions.

In view of the conflicting nature of the phenomena requiring explanation, it is probable that the relationship between the mortality rates is not due to a direct causal connection between tuberculosis and cancer.

This receives major support from the fact that the relationships have been based upon the "crude" and not the age-standardised mortality rates. As the

populations of both England and Australia have been ageing during the period of investigation, at least some of the increase of cancer mortality must be attributed to this age change. The age standardised mortality rates for Britain (Hammond, 1958) and Australia (Lancaster, 1958) show only slight rises in the cancer mortality for males and a little or no rise for females. In England and Wales between 1931 and 1947 female death rates from tuberculosis fell by one third to one half at every age and those from cancer fell by about one tenth at every age between 35 and 75. Male tuberculosis rates in the same period fell by one half at ages under 45 but remained unchanged at ages over 65. Their cancer rates have risen because of more complete recognition, coupled with a real increase in lung cancer ; when lung cancer is excluded the rates for other cancer have been falling since 1931 at ages under 70. If the population had not changed, the joint tuberculosis and cancer mortality rate would not have remained constant.

More direct observations have failed to substantiate a causal relationship between tuberculosis and cancer, although much of this work is of doubtful value. Grosse (1959) has shown that it is fallacious to determine associations from uncorrected autopsy statistics, a method used in a comprehensive study by Pearl (1929). This purported to show that compared with controls, florid, active tuberculosis was infrequent in persons dying with malignant growths and *vice versa*.

A more reliable approach is to examine the mortality experience of a specified group of tuberculous patients. This method has shown that apart from lung cancer, tuberculous patients do not have a significantly different mortality from cancer than the general population (Campbell, not yet published).

Although it is necessary to reject a causal relationship as an explanation of the mortality relationships of tuberculosis and cancer, their striking consistency suggests that they are not merely fortuitous and that some indirect relationships may be operative. The constancy of the tuberculosis and cancer joint total of deaths over a period of years before modern tuberculosis treatment began to accelerate the fall in mortality from that disease could be due to a group of factors which happened to have equal and opposite effects on the total deaths from tuberculosis and cancer.

Excepting recent years, the improvement of the tuberculosis mortality can be attributed to improved socio-economic conditions. Similarly, the ageing of the population has probably been brought about by the same improved conditions. Assuming that these improvements have not reduced the total impact of carcinogenic factors, the following relationship can be postulated.

		Effect on crude death rate from	
		Tuberculosis	Cancer
a. Improving social conditions (food, housing, etc.)	→ Better nutrition	−	..
	→ General population live longer	..	+
b. Improving health services	→ Prevention of infective diseases	−	..
	→ More early treatment	−	−
	→ Better diagnosis of cause of death	..	+
c. More urbanisation, industrialisation and smoking	→ More inhalation of pollutants and contact with carcinogens	+	+

Within a developing country the national crude death rates from tuberculosis and cancer will move with time in opposite directions through the combined operation of factors (*a*), (*b*) and (*c*). However, rate differences between parts of the country (e.g. urban and rural) will arise chiefly from (*c*) and will be parallel for the two diseases; and for rate differences between countries with similar levels of social conditions and health services this will also be true. Between countries at very different stages of development the effects of (*a*) and (*b*) might outweigh those of (*c*) and produce opposing differences in respect of the crude tuberculosis and cancer rates.

Although the various mortality relationships do not establish a causal relationship between tuberculosis and cancer, they deserve further consideration, if only to suggest further lines of enquiry.

SUMMARY

1. The observations of earlier investigators concerning the relationship between the crude death rates of all types of neoplasm and tuberculosis have been reviewed and largely confirmed. It has been shown that for 50 years the joint mortality rate from tuberculosis and all types of cancer has remained remarkably constant for the Australian population as a whole and the male and female population separately. The Queensland joint mortality rate has also remained relatively constant. No other pair of major diseases have behaved similarly. In contrast to the mortality relationships within a locality, localities differ from each other by parallel differences in the mortality rates; a high tuberculosis rate is associated with a high cancer rate.

2. In the past, these observations have been considered to lend support to a direct causal relationship between the two diseases and the various theories have been discussed.

3. Owing to the effect of ageing of the population upon crude cancer death rate, the rise in cancer mortality is not due simply to the fall in tuberculosis mortality and a direct causal explanation of the mortality relationship has been rejected.

4. It has been suggested that improved social welfare and health services contribute to the decrease of tuberculosis and at the same time lead to ageing of the population. This, in turn, increases the crude cancer rate. Provided these changes have approximately equal and opposite effects then the joint mortality rates would remain constant.

5. The parallel differences in cancer and tuberculosis mortality according to locality and occupation can arise from conditions associated with urbanisation and industry which should favour the development of both tuberculosis and cancer.

Thanks are due to Mr. S. E. Solomon, Government Statistician, and his staff of Queensland for tuberculosis and cancer mortality rates in Australia and Queensland, and to Mr. A. G. Pickford who drew the illustrative figures.

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CANCER OF THE LUNG IN SOUTH-WEST ENGLAND AND LONDON: AN EPIDEMIOLOGICAL STUDY OF HISTOLOGICAL TYPE

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THE importance of distinguishing between the various histological types of lung cancer has been stressed in many epidemiological studies. It has been suggested for example that cigarette smoking is associated with squamous and anaplastic rather than with glandular celled tumours. There are not, however, any statistics available on a national basis which make this distinction. Under the Registrar General's National Cancer Registration Scheme (see, for example, Stocks, 1950) information is collected concerning a proportion of all cases of malignant disease diagnosed throughout England and Wales. This proportion was believed to be about 55 per cent in 1952 (Ministry of Health, 1956). Although histological descriptions are not reported, the existence of the scheme makes it possible to identify all cases of lung cancer recorded in the participating hospitals and thus to collect details of the histology and any other factors recorded locally.

The original purpose of the present study was to identify all cases of lung cancer registered in the South Western Hospital Region of England during the 12 years 1945–56 for which a histological diagnosis had been made, and to determine the proportions of the main histological types of lung cancer among various groups of the population. The South Western Region was chosen because it was believed to have had, throughout the period, a registration coverage higher than that of the whole country and because its geography made it unlikely that many patients living within its boundaries would go outside them for medical care. The accumulated records for such a region should provide a reasonably unselected series of patients—in contrast to some highly selected series described in published reports.

The proportion of cases histologically described was considerably lower than expected. Although 60 per cent of South Western Regional cases were reported as confirmed by pathological examination, in only 40 per cent of those records studied was there a record of microscopical diagnosis. This finding persisted throughout the 12 years of the survey and meant that the cases under review, which represented one fifth of the total lung cancer deaths in the region in 1945 had only increased to represent one-third of the deaths in 1956. The selection implied in these proportions nullified the advantage implicit in the nearly complete overall registration. The original aim of confining the study to a single geographical region was accordingly abandoned and similar material was collected from two London teaching hospitals, St. Bartholomew's and the London Hospital.

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METHOD OF INVESTIGATION

The material for study consists of 3965 cases of cancer of the lung and bronchus registered during the years 1945–56 in the South Western Hospital region and in the two teaching hospitals. The information, which was derived from a personal study made by one of us (M. H.) from the hospital case-notes, was collected under the following headings :

Age and sex
Year of diagnosis
Histological diagnosis
Method of diagnosis
Residence (town or country)

Histological diagnosis

Cases were accepted for analysis only if there was available a report, *in the original words of the reporting pathologist*, of the microscopic appearances either of a tumour section (surgical or post mortem) or of biopsy material. In such a large series the reports were inevitably made by several different pathologists and a uniform and accurate system of classification was out of the question. All that was attempted was to label each case according to one of the following headings :

Squamous cell carcinoma, including all growths described as squamoid or epidermoid without further qualification.

Anaplastic carcinoma, including all undifferentiated growths unless described as " oat-cell carcinoma ".

Oat-cell carcinoma, including only tumours described as such.

Adenocarcinoma, including columnar cell carcinoma, malignant adenoma and alveolar cell carcinoma.

It was originally intended to keep malignant adenoma and alveolar cell carcinoma separate from adenocarcinoma. There were however several cases in which, from the description given, the distinction could not be made, and it was decided to keep all three tumours in one group, corresponding to the Group II of Kreyberg (1952, 1954). Of the cases studied there were about 5 per cent which could not be fitted into this clasification either because they were described simply as " bronchogenic carcinoma " or because they were mixed types such as " adeno-squamous ". These are not included in the analysis.

Method of diagnosis

The methods of diagnosis were classified as resection, post mortem, or biopsy. The term biopsy is taken, for the purposes of this report, as including the cytological examination of the sputum as well as histological examination of specimens obtained at thoracotomy, bronchoscopy and skin or lymph node excision.

Residence

In view of the many reports of different rates of mortality from lung cancer in town and country, the patients' home addresses were recorded. This information was classified according to the size of the population in the town or area of residence (Table II).

RESULTS

Of the total of 3965 cases, just over half (2079) were from the South Western Region. Of the remainder, 1174 were registered at the London and 712 at St. Bartholomew's Hospital. In 1892 cases the diagnosis was based on a tumour section (operation, 1269 ; post mortem, 623), the source of the remainder, grouped under the heading " biopsy ", being as follows :—

Bronchoscopy	1150
Thoracotomy	293
Gland or skin biopsy	342
Needle biopsy	91
Sputum examination	197

The distribution of the 4 histological types according to various factors are shown in Table I and II. It can be seen immediately that there are considerable differences between the sexes. The proportion of squamous cell tumours was much lower among women (24 per cent) than among men (49 per cent) and the proportion of the other types of tumour were correspondingly higher. In particular the proportion of adenocarcinoma was considerably higher among women than men (20 per cent compared with 10 per cent). The male-female ratios for the four types of tumour were :—

Squamous cell	16·5 : 1
Anaplastic	6·5 : 1
Oat-cell	5·7 : 1
Adenocarcinoma	4·0 : 1

In view of these differences the sexes have been separated throughout the subsequent analysis and the greater part of the following relates to men only.

The distribution of histological types varies considerably according to the method of diagnosis. Considering the males only it can be seen from Table I that

TABLE I.—*Histological Type by Sex and Method of Diagnosis*

		Total	Histological type			
			Squamous	Anaplastic	Oat-cell	Adeno-carcinoma
Males	Number	3535	1733	806	658	338
	Per cent	*100*	*49*	*23*	*19*	*10*
Resection	Number	1186	740	191	138	117
	Per cent	*100*	*62*	*16*	*12*	*10*
Post mortem	Number	517	129	138	162	88
	Per cent	*100*	*25*	*27*	*31*	*17*
Biopsy	Number	1832	864	477	358	133
	Per cent	*100*	*47*	*26*	*20*	*7*
Females	Number	430	105	124	116	85
	Per cent	*100*	*24*	*29*	*27*	*20*
Resection	Number	83	30	13	14	26
	Per cent	*100*	*36*	*16*	*17*	*31*
Post mortem	Number	106	13	27	33	33
	Per cent	*100*	*12*	*25*	*31*	*31*
Biopsy	Number	241	62	84	69	26
	Per cent	*100*	*26*	*35*	*29*	*11*

more than half the operation specimens (62 per cent) were described as squamous cell tumours, the order of frequency of the others being anaplastic (16 per cent), oat-cell (12 per cent), and adenocarcinoma (10 per cent). Among post mortem specimens, however, the position was almost reversed with oat-cell the most frequent type of tumour (31 per cent), followed by anaplastic (27 per cent).

TABLE II.—*Histological Diagnosis by Centre, Age and Domicile*

	Total	Histological type (percentage)			
		Squamous	Anaplastic	Oat-cell	Adeno-carcinoma
Males					
Centre					
S-W. Region	1856	49	22	18	11
St. Bartholomew's Hospital	635	50	21	19	10
London Hospital	1044	48	25	19	8
Age					
Less than 35	44	27	27	41	5
35–	78	33	21	32	14
40–	195	42	19	25	14
45–	402	43	24	24	8
50–	671	52	20	20	8
55–	780	50	24	15	10
60–	683	52	23	16	9
65–	420	49	24	18	9
70–	190	50	26	14	10
75–	60	53	23	13	10
Residence					
Towns with pop. 100,000 or more	1839	48	21	20	10
Towns with pop. 50,000–100,000	315	48	24	16	12
Towns with pop. less than 50,000	770	49	24	18	9
Rural districts	456	51	23	17	9
Females					
Centre					
S-W. Region	223	23	31	26	20
St. Bartholomew's Hospital	77	25	23	32	19
London Hospital	130	26	28	26	19
Age					
Less than 45	72	15	31	35	19
45–	119	18	35	25	21
55–	145	31	23	27	19
65–	92	28	28	23	21
Residence					
Towns with pop. 100,000 or more	240	21	28	29	21
Towns with pop. 50,000–100,000	113	28	29	25	18
Towns with pop. less than 50,000					
Rural districts	63	22	32	25	21

Note : The age was unknown in 14 cases and the place of residence in 169 cases.

squamous cell (25 per cent) and adenocarcinoma (17 per cent). The distribution of biopsy diagnosis occupied an intermediate position except that the proportion of adenocarcinoma (7 per cent) was even lower than that among resection specimens.

These findings are clearly related to the site, rate of growth, tendency to metastasize and other factors governing the operability of different types of tumour (for example adenocarcinomas are often peripheral growths which are not accessible to bronchoscopic biopsy). Consideration of these factors was outside the scope of this survey.

In Table II are shown the distributions of the four histological types according to centre of registration and the age and domicile of the patient. Among men anaplastic tumours and adenocarcinomas did not show any consistent variation with age but there was a steep decrease in the frequency of oat-cell carcinoma with increasing age and a less well marked increase in squamous cell growths. This finding is discussed in a later paragraph. The area of residence did not appear to be related to the histological type.

When this survey was planned it seemed possible that we might be able to throw light on the hypothesis that, over a number of years, the proportion of adenocarcinomas to all types of tumour was decreasing. Table III shows that the

TABLE III.—*Method of Diagnosis by Year* (*Males, all Histological Types*)

	Total	Method of diagnosis (per cent)		
		Resection	Post mortem	Biopsy
1945–46	104	*11*	*11*	*79*
1947–48	254	*17*	*14*	*69*
1949–50	480	*22*	*18*	*60*
1951–52	733	*34*	*16*	*50*
1953–54	893	*39*	*18*	*42*
1955–56	1057	*40*	*10*	*50*

Note : The year was not recorded in 14 cases.

total number of cases per year in the series increased ten-fold from the first to the last two years of the period. This reflects not only the increasing efficiency of the registration system but also an increase in the proportion of those cases seen in hospital in which a histological diagnosis was made. This fact alone makes it unlikely that any firm conclusion can be drawn from a study of time-trends for the different types of tumour, as the factors governing the selection of cases are almost certain to have changed. In particular there were, as Table III shows, considerable changes in the proportions of cases diagnosed by the three different methods. These may, of course represent real changes in practice : an increase, for example, in the proportion of diagnoses made at operation would be expected, but hardly of the extent shown here. When studying the proportions of histological types these changes can be allowed for by considering each method of diagnosis separately, remembering that no account can be taken of changes *within* the methods, brought about, for instance, by changing standards of operability.

None of the time-trends for the four different types of tumour (Table IV) show any consistent pattern, with the possible exception of an increase in anaplastic at the expense of oat-cell tumours. It seems probable that most of the fluctuations in the rates are due to vagaries in the method of selection and, in the early years, to the small number of cases.

TABLE IV.—*Histological Type by Method of Diagnosis and Year (Males)*

	Total	Histological type (per cent)			
		Squamous	Anaplastic	Oat-cell	Adeno-carcinoma
Resection					
1945–46 . .	11	67	9	19	5
1947–48 . .	43				
1949–50 . .	107	67	11	10	11
1951–52 . .	246	61	19	9	11
1953–54 . .	351	58	20	11	11
1955–56 . .	424	66	13	13	8
Post mortem					
1945–46 . .	11	17	23	38	21
1947–48 . .	36				
1949–50 . .	84	20	23	39	18
1951–52 . .	117	29	23	32	16
1953–54 . .	163	28	31	26	15
1955–56 . .	104	18	33	32	18
Biopsy					
1945–46 . .	82	51	18	26	5
1947–48 . .	175	54	13	31	2
1949–50 . .	289	53	22	19	6
1951–52 . .	370	48	23	19	10
1953–54 . .	379	49	33	17	6
1955–56 . .	529	43	31	17	9

TABLE V.—*Proportions of Oat-cell Tumours to all Anaplastic Tumours (including Oat-cell): Males: Resection only*

	(Per cent)			
	1945–50	1951–52	1953–54	1955–56
Age				
Less than 55 . .	59	47	40	58
55 or more . .	50	23	31	41

Oat-cell tumours

Not all pathologists agree that oat-cell tumours form a group distinct from other anaplastic tumours, and in many published series the two are combined. For this reason our finding that the oat-cell tumour is the only type whose relative frequency is at all strongly associated with age is particularly interesting. In fact Bryson and Spencer (1951) made a similar observation in a series of 866 autopsies; they found the mean age of patients with an oat-cell tumour to be 3 years younger than that of "polygonal cell growths" but they did not comment on the finding.

The age distributions for the 4 types of tumour and three methods of diagnosis are shown in Fig. 1. They show clearly the consistent way in which oat-cell tumours are distinguished from the remainder. The difference was most marked among operation specimens, where the median age was approximately 4 years younger in oat-cell than in the other tumours. This age difference appeared consistently when the material from each centre was analysed separately.

During the period of this survey there was a tendency for the proportion of older patients to increase. The younger patients therefore include relatively more

from the earlier years than do the older. If, as is possible, the term " oat-cell " has become less widely used by pathologists over the period, the result would be an apparent association between oat-cell tumours and younger patients. In fact this is not the cause of our finding as may be seen by studying the proportions for shorter periods of time. Table V shows the proportions of oat-cell tumours, calculated from all anaplastic (including oat-cell) tumours, in operation specimens over four such periods.

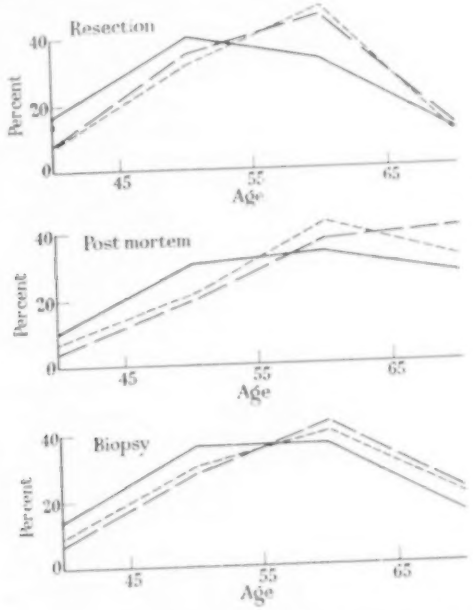

FIG. 1.—Age distribution of males in 10 year age groups:
Squamous cell carcinoma — — —
Anaplastic carcinoma - - - - -
Oat-cell carcinoma ———

In each period the oat-cell ratio was distinctly higher in younger than in older patients. Calculation of oat-cell ratios for the same periods of time for biopsy and for post mortem diagnosis gave the same results. It is difficult to think of any reason, unrelated to the appearance of the tumours, which would consistently lead pathologists from various parts of the country, and over a period of ten years, to agree in associating the term " oat-cell " with younger patients, regardless of the method of diagnosis. There would seem to be a real distinction between oat-cell and other anaplastic tumours.

Females

The smaller number of women does not permit such detailed analysis as is possible for men. A few points do nevertheless emerge from a study of the female

TABLE VI.—*Percentage Frequency of Histological Types: Comparison with Other British Series*

		Total	Histological type (per cent)			
			Squamous	Anaplastic	Oat-cell	Adeno-carcinoma
Resection						
Giffard and Waddington (1957)						
1941–54	M.	318	56	34		10
	F.	29	35	52		13
Belcher (1956)	M., F.	264	66	16		18
1948–54						
Walter and Pryce (1955)	M., F.	201	61	8	16	16
1950–53						
Present series	M.	1186	62	16	12	10
	F.	83	36	16	17	31
Post mortem						
Galluzzi and Payne (1955)	M.	611	26	62		12
1948–52	F.	129	15	68		17
Walter and Pryce (1955)	M., F.	153	21	29	39	11
1948–53						
Bryson and Spencer (1951)	M.	740	19	41	37	3
1936–47	F.	119	14	38	34	13
Present series	M.	517	25	27	31	17
	F.	106	12	25	31	31
All methods						
Doll and Hill (1952)	M.	811	59	37		4
1948–52	F.	66	27	58		15
Present series	M.	3535	49	23	19	10
	F.	430	24	29	27	20

In some instances percentages have been recalculated, omitting the unclassified cases.

data. Table I shows that although fewer squamous cell and correspondingly more of each of the other types of tumour were diagnosed among women, there was a pattern similar to that among men for the methods of diagnosis. For example the highest proportion of squamous cell tumours was among resection specimens, while post mortem material contains high proportions of oat-cell tumours and adeno-carcinomas.

The figures also show that only 19 per cent of diagnoses among women, compared with 34 per cent among men were made on resected specimens; post mortem examination accounted for 25 per cent of the diagnoses in women as against 15 per cent for men. These observations might suggest that there were more older women than men in the series. In the series as a whole the women were in fact slightly younger than the men (45 per cent under 55 years of age compared with 39 per cent). The difference was particularly marked in the very young patients as 6 per cent of the women and only 1 per cent of men were under 35 years

of age. Of the 25 women under 35 years of age as many as 13 (52 per cent) had oat-cell tumours. Apart from this finding (which may be completely fortuitous) there were no striking variations in the age distributions between the four histological types. There is, however, a suggestion that squamous cell tumours were slightly more common among older women (in spite of the fact that these tumours are found more often at operation) and anaplastic more common among younger patients. A study of time-trends and the distribution according to urbanisation of place of residence yielded nothing of interest.

DISCUSSION

If a histological diagnosis were available for all cases of lung cancer a complete national registration scheme would provide the ideal basis for the study of the epidemiology of the different histological types ; it would hardly then be necessary to consider the source of the material whether from autopsy, biopsy or resection specimens. Unfortunately it seems that histological data are available for considerably less than half the patients with diagnosed lung cancer in this country. In the early years of Cancer Registration Scheme it was reported that 38 per cent of cases of male lung cancer were proved histologically (Stocks, 1950), but there is reason to suppose that this proportion included cases subjected to no more than a naked-eye examination and that the true figure was, at that time, not much above 30 per cent.

The present series of histologically diagnosed cases probably represents less than a third of all the cases occurring in the South Western Region together with about three quarters of all the cases referred from an indeterminate area to two London teaching hospitals. It cannot therefore be claimed to be free from bias. The large numbers, however, make it possible to allow for some, at least, of the sources of bias by enabling the data to be sub-divided according to several different factors.

A particular difficulty which has been made much of by some writers, for instance Walter and Pryce (1955), is that there is wide variation in criteria of diagnosis among different pathologists. In fact, if we restrict comparisons to this country and make allowances for sex and method of diagnosis the differences between the proportions reported from different centres are remarkably small. This is borne out by the comparisons made within our series and with those of some of the well known British series as shown in Table VI. The Table shows, for example, that there is general agreement that women have fewer squamous cell tumours and more adenocarcinomas than men whatever the method of diagnosis and also that the proportion of undifferentiated tumours is consistently greater in necropsy than in other material.

In spite of their limitations we believe that histological data based on cases registered under the National Cancer Registration Scheme do provide a potentially useful source of information in the epidemiological study of lung cancer. Apart from the bare statement of the proportions of histological types by sex and method of diagnosis our one positive finding has been to confirm that of Bryson and Spencer (1951) that men with oat-cell tumours are, on the average, younger than those with other anaplastic growths. We have already discussed our reasons for believing that this reflects a real difference between the two types of tumour and we would like to echo the plea of Walter and Pryce (1955) that the term " oat-cell "

should be retained and that these tumours should be counted separately in studies of this sort.

Our chief negative result has been that we have been unable to detect any association between cell type and place of residence in whatever way the material was analysed. In view of Kreyburg's (1956) finding of a higher incidence of squamous cell cancer among townsmen than among countrymen it is perhaps suprising that no such differences were found in this material. There is no apparent reason why the selective factors at work in this series should have masked this particular relationship and we only conclude that, if present at all in this country, it is not as well marked as in Norway.

We were able to produce no real evidence, positive or negative, on the question of the changes in the relative proportions of different cell types over the period reviewed. Spain (1959) has recently reviewed the autopsy material in a New York hospital and shown that, relative to adenocarcinoma, squamous and anaplastic growths have increased more than four-fold over a period of forty years.

SUMMARY

The paper describes a study of the histological diagnosis in 3965 cases of lung cancer registered under the National Cancer Registration Scheme in the South-West of England and in London in the years 1945–56.

The distribution of the main histological types was found to vary considerably according to sex and method of diagnosis (operation, biopsy and post mortem). The findings were similar to those of other workers from this country.

The proportion of " oat-cell " tumours was found to decrease with increasing age (from 30–40 per cent at ages less than 40 to about 15 per cent at ages above 55). This was not true of other anaplastic growths and reasons are given for believing that the finding reflects a real aetiological difference between oat-cell and other anaplastic growths.

No conclusions were drawn from a study of the variations over time in the proportions of the histological types and no relationship was found between the histological type and the area of domicile (town or country).

The advantages and disadvantages of a survey based on the National Cancer registration scheme are discussed.

This survey could not have been undertaken without the active co-operation of many people, but acknowledgment should be made especially to the late Professor Sir Ernest Kennaway under whose guidance it was initiated ; to Mr. R. M. Vick and the staff of the South Western Regional Cancer Records Bureau ; to Dr. W. P. D. Logan and other members of the General Register Office ; to Professor J. W. S. Blacklock, Professor Dorothy Russell and other pathologists at St. Bartholomew's and at the London Hospital ; and to Dr. Richard Doll.

We are also grateful for the help given by the staffs of the records departments in the contributing hospitals and the Department of Medical Statistics at St. Bartholomew's Hospital where the material was analysed on punched cards.

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CANCER OF THE LIVER, PANCREAS AND PERITONEUM IN SINGAPORE

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THIS paper discusses, from two standpoints, malignant tumours of the liver, pancreas and peritoneum in Singapore. Firstly, the incidence and morbid anatomy of such tumours, seen in the post mortem room by the staffs of the University and Government Departments of Pathology, in the 22,997 autopsies carried out in the years 1948–58 inclusive, are described. Secondly, the morbidity and mortality from these cancers, derived from hospital and Registrar General's figures, for the years 1954–58 inclusive, are considered. This study is complementary to a previous review of gastro-intestinal tract tumours (Muir, 1959).

The age, sex, and racial structure of the Singapore population for this period have already been described (Muir, 1959) and some of the possible sources of error and bias in the post mortem material and in the records of the Registrar General (Singapore) have been indicated. Briefly, half the Singapore population, both living and post mortem, is under 21 years of age, and while the relative racial proportions for the population as a whole are: Chinese, 76·3 per cent; Malay, 13·1 per cent; Indians and Pakistanis, 8·1 per cent; and Others 2·5 per cent, the corresponding post mortem figures are 87·0 per cent, 2·6 per cent, 8·4 per cent, and 2·0 per cent. Some one-third of deaths are registered by hospital assistants and police officers.

The records of nearly all post mortems performed on the Island in this period were available to the author. These were scrutinised individually and the relevant data entered on a proforma for subsequent analysis.

LIVER

In all, there were 189 malignant tumours falling within the scope of International List No. 155, i.e., tumours of the ampulla of Vater, the biliary passages (both intra-hepatic and extra-hepatic), the gall bladder, and the liver itself.

Six of these were situated in the gall bladder. Five were in males. The mean age at death for the six was $58{\cdot}2 \pm 6{\cdot}0$ years. Spread to the liver and to the regional nodes was seen in 4 instances; metastasis to the lungs, adrenals, peritoneum and stomach was noted in one case. Two of the livers showed cholangiectasis with abscess formation; choleliathiasis was seen once.

A further five tumours, all in males, were found in the extra-hepatic bile ducts. The mean age at death was $46{\cdot}8 \pm 8{\cdot}6$ years. Two of these cancers arose in the common bile duct near the point of junction of the hepatic ducts, another in the left hepatic duct, and in the remaining two the exact point of origin was in doubt. Cholangiectasis was present in all five, calculi in four, biliary cirrhosis in two.

Secondary spread to the regional lymph nodes was seen in all five; widespread metastasis to lung, liver, omentum, suprarenal, and heart, in two.

The remaining 178 were within the liver itself. One hundred and sixty-four of the tumours were found in males whose mean age at death was 48·6 ± 10·6 years, and 13 in females, the mean age at death being 44·5 ± 11·9 years. (A female aged 1 year is excluded from this figure.) The remaining cancer, a malignant haemangio-endothelioma, was discovered in a Chinese male aged 19 years.

Five of these primary liver tumours were seen in Indian males, whose mean age at death, 34·6 ± 8·1 years, was thus appreciably lower than that of the Chinese males.

The pattern of age distribution of the 178 cases is shown in Table I. It will

TABLE I.—*The Age Distribution, by Sex, of 178 Singapore Cases of Primary Liver Tumours*

Age	Males		Females		Total	
	Number	Per cent	Number	Per cent	Number	Per cent
0–9	—	—	1	8	1	1
10–19	3	2	—	—	3	2
20–29	5	3	1	8	6	3
30–39	18	11	3	23	21	12
40–49	64	39	4	31	68	38
50–59	47	28	2	15	49	28
60–69	25	15	1	8	26	15
70 +	3	2	1	8	4	2
Totals	165	100	13	101	178	101

be noted that there were more deaths before the age of 50 than there were after. Liang and Tung (1959) tabulate the age distribution of primary liver cancers for three series: 258 Chinese, 736 Europeans and Americans, and 569 Africans. In China 83 per cent of deaths occurred before the age of 50; in Europe and America, 32 per cent; in Africa, 93 per cent. The peak incidence in China, as in Singapore, was in the decade 40–49.

Grossly, the vast majority of livers were enlarged. The mean weight for the male being 2800 g., for the female, 2300 g. The least weight recorded was 870 g., the greatest 7700 g. The tumours were found to be principally in the right lobe in 55 per cent, mainly in the left lobe in 21 per cent, being distributed more or less uniformly throughout in 21 per cent. The site was not stated in the remaining 3 per cent.

A solitary tumour was seen in 14 per cent. In 34 per cent satellite nodules of tumour were seen round the main mass, and in 20 per cent, as well as the principal tumour, nodules were found scattered throughout the remainder of the liver. In 29 per cent the nodules were multiple, no one being sufficiently larger than its fellows to warrant the title of principal tumour.

The solitary or principal tumours were usually about 12 cm. in diameter. They were generally soft and pulpy, green, reddish, and yellow-white in colour depending on the extent of necrosis, biliary impregnation and haemorrhage. The smaller nodules, 1 to 3 cm. in diameter, were usually white in colour, although a variegated appearance was seen from time to time. On occasion the nodules blended imperceptibly into the surrounding liver tissue. In some 5 per cent

umbilication of small sub-capsular nodules was noted. If the main tumour mass reached the capsule, it tended to grow in an arboreal, or fern-like manner for some time beneath the capsule before penetration. Often the solitary or principal tumour protruded well beyond the liver, usually from the right lobe, down towards the pelvis. One such was 16 cm. × 12 cm. × 12 cm. in size.

Spread along the blood vessels of the liver was extremely common. In 24 per cent the portal vein was blocked with tumour as far as the junction of the major portal branches, and continued outside the liver to the lesser omentum in a further 22 per cent. The splenic vein was involved in 5 per cent, the mesenteric vein in 3 per cent and the gastric vein in 2 per cent. Doubtless due to the impeded venous return from the spleen, in many, to a pre-existent cirrhosis, splenomegaly was common, being observed in 35 per cent. The largest spleen weighed 670 g., the mean weight being 340 g.

Spread along the hepatic veins was much less frequent, being found in 6 per cent. In a further 4 per cent the tumour extended into the inferior vena cava, and in another 2 per cent into the right atrium.

Metastasis within the abdominal cavity was infrequent. The diaphragm was involved, principally by direct extension, in 7 per cent; the greater omentum in 6 per cent; the lymph nodes of the porta hepatis, in 8 per cent: the para-aortic nodes in 5 per cent; spread to the stomach was seen in 3 per cent; to the kidney and adrenals in 2 per cent.

The lungs showed secondary tumour in 28 per cent. These were usually bilateral, very often sub-pleural, and of the cannon ball type. In some 4 per cent worm-like tumour casts could be expressed from the pulmonary arteries. Metastases were seen in bone and brain in 3 per cent of cases.

Oesophageal varices were noted in 15 per cent, and in 9 per cent one or more of these had ruptured with significant haemorrhage into the gut.

Ascites were present in 25 per cent, the majority showing at least a tinge of blood staining. Twenty-four per cent exhibited rupture of the tumour with consequent haemorrhage into the abdominal cavity. This varied considerably in amount, 1·5 litres being a not uncommon quantity. In the brief clinical histories accompanying the post mortem notes there was no mention of injury in any case, but in two persons the haemorrhage followed percutaneous aspiration biopsy.

Brown atrophy of the heart was noted in 15 per cent and seemed particularly common in those under 45 years of age.

The morbid anatomy corresponds, in the main, fairly closely to that of prior Singapore series of primary liver cancers (Tull, 1932; Shanmugaratnam, 1956), although Tull (1932) found portal vein invasion in but 20 per cent, and intra-abdominal haemorrhage in but one of 134 cases.

THE PANCREAS AND PERITONEUM

There were 20 cases of pancreatic carcinoma. Fifteen were in Chinese males, whose mean age at death was 49·9 ± 7·5 years. Of the remainder, three were Chinese females, one an Indian male, one a Eurasian female. The mean age at death for the 20 was 52·5 ± 7·8 years.

The tumours were usually of the scirrhous type, rock-hard to the touch. Two were described as mucoid, one of which was a multiloculate cystadeno-

carcinoma. Thirty-five per cent were found in the head, 30 per cent encroached on both head and body, 20 per cent were in the body while 15 per cent extended over most of the organ.

Secondary spread to the lung was found in 30 per cent ; to the liver in 65 per cent ; to the brain, bones, and kidneys in 10 per cent. Peritoneal dissemination was seen in 20 per cent ; regional lymph node involvement in 70 per cent. The stomach and duodenum were directly invaded in 30 per cent while the extra-hepatic bile ducts were either narrowed or closed in only 30 per cent.

There were no primary tumours of the peritoneum at post mortem. Although 18 instances were registered these were probably secondary to malignancy elsewhere.

MORTALITY AND MORBIDITY

The 178 primary liver neoplasms represent 0·77 per cent of all post mortems carried out in the 11 years under review, or, if the 11,525 children under the age of 11 years be omitted, 1·55 per cent, or, 16·2 per cent of the 1096 malignant tumours seen in the mortuary.

Liver cancer (List No. 155–56) accounted for 759 of the 5915 deaths from malignant disease recorded by the Registrar General (Singapore) in 1948–58, i.e. 12·8 per cent. In view of the post mortem prevalence of primary liver cancer the author had assumed that the bulk of these tumours would fall within List No. 155. By the kindness of the Singapore Government Department of Statistics, who provided, for 1957 and 1958, from their punched card records a more detailed breakdown of malignant tumours by site, sex, and five-year age group, this premise was shown to be false, only 13 per cent of all malignant liver tumours for the two years being entered under List No. 155. A closer scrutiny of this fuller analysis showed that, in fact, in 1957 and 1958 there were more primary carcinomata of the liver at autopsy (41) than there were registered (26). This would indicate a breakdown in the coding system.

With this in mind the grouping together of List No. 155 and 156, and the calculation of age specific death rates for cancer of the liver based on this aggregate, was considered justified. These are given in Table II. Those for 1957 are related to the 1957 census population, and those for 1957 and 1958 combined are again related to the same population, the larger numbers so obtained tending to smooth out any irregularity of age distribution from year to year. For comparison similar rates are given for malignant neoplasms as a whole (List No. 140–205).

If it be assumed that there were, in fact, few secondary liver cancers, then in 1954–58 primary liver neoplasms were nearly as common as bronchial carcinomata, and about half as frequent as gastric cancers. In the 11-year post mortem review the relative proportions of primary cancers of the liver, lung and stomach were 1·00 : 0·92 : 0·97 ; in 1954–58 the proportions were 1·00 : 1·2 : 1·07.

In the eleven years there were 839 admissions to hospital with a liver cancer (List No. 155–156) of whom 354, or 42 per cent, died there. With 189 post mortems the autopsy rate was thus 53 per cent.

For the years 1948–53 inclusive the hospital returns included carcinoma of the pancreas under "other digestive organs". From 1954 onwards it has been entered as a disease in its own right under International List No. 157. In the quinquennium 1954–58 there were 64 admissions to hospital with 28 deaths.

In the same period of time the Registrar General (Singapore) recorded 37 deaths ; 13 (35 per cent) of the cases registered in the five years came to post mortem. The crude incidence rate for both sexes is 0·5 per 100,000 per annum. In view of the small numbers, age specific death rates are not given ; however, an age adjusted figure is to be found in Table III.

The crude death rate from cancer (List No. 140–205) in the quinquennium 1954–58 was 51·6 per 100,000 living per annum, from liver cancer (List No. 155–156) 6·3 per 100,000, and from malignancies of the digestive organs and peritoneum (List No. 150–159) 25·0 per 100,000. Thus these latter cancers comprised 48·5 per cent of all malignant tumours registered. Virtually comparable figures, for England and Wales, Japan and Australia, are given by the World Health Organization (1956). In the triennium 1951–53, for males these were 46·0, 85·1, and 49·5 per cent ; for females, 44·9, 60·4 and 43·3 per cent. In Singapore, in 1957–58, 55·8 per cent of male malignancies registered, and 38·5 per cent of the female, arose in the digestive system and peritoneum.

It is held that the comparison of tumour incidence between countries is facilitated by the use of age adjusted death rates. Such adjustments are usually made to the population of the writer's own country. Segi (1960) has derived a standard population based on the age constitution, in 1950, of 46 widely scattered countries, and it is to this hypothetical standard population that adjustment has been made in Table III. It will be noted that as in Table II, a figure has been given for Singapore, for 1957, and for the mean incidence in 1957–58, both based on the 1957 census population. The values for England and Wales, Japan, and Australia, based on the same standard population, are taken from Segi (1960). The figures speak for themselves.

This type of comparison may be criticised if the figures for a relatively small population are adjusted to those of a much larger. To circumvent this in some measure, the mean number of cases observed in the Singapore populace in 1957–58 is compared with the number expected for the populations of England and Wales, Japan, and Australia (Table IV). In the calculation of these expected numbers,

TABLE II.—*Age Specific Death Rates, per* 100,000 *Living per annum, for Liver Cancer (List No.* 155–156) *and for All Cancers (List No.* 140–205)

	Liver cancer						All cancer					
	1957			1957–58			1957			1957–58		
Age	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
0–4	—	—	—	—	—	—	7·3	5·5	6·4	5·9	5·1	5·5
5–9	—	—	—	—	—	—	2·7	3·8	3·2	3·1	2·8	3·0
10–14	—	—	—	0·7	—	0·4	4·2	—	2·2	7·7	3·1	5·5
15–19	1·4	—	0·7	0·7	—	0·4	11·4	4·6	8·1	9·3	3·8	6·6
20–24	—	—	—	—	—	—	3·3	3·4	3·3	5·7	4·3	5·0
25–29	3·3	3·8	3·6	2·5	1·9	2·2	10·0	9·6	9·8	16·7	14·4	15·7
30–34	—	—	—	7·0	—	3·9	21·9	30·2	25·6	25·9	22·6	24·5
35–39	12·9	—	7·3	8·6	2·7	6·0	48·3	55·2	50·8	50·5	60·8	55·0
40–44	11·6	3·0	7·9	17·4	1·5	10·5	83·6	93·4	87·9	94·1	85·9	90·5
45–49	45·0	3·4	27·0	45·0	6·9	28·4	185·3	120·9	157·4	202·5	127·8	170·1
50–54	51·2	17·7	36·7	49·6	8·9	31·9	263·2	199·6	235·5	261·5	179·7	225·9
55–59	48·7	11·8	32·0	48·6	23·7	37·4	384·4	266·2	331·1	403·9	248·4	333·7
60–64	83·2	48·9	65·9	74·9	40·8	57·7	532·7	269·1	399·6	566·0	289·5	426·4
65–69	147·9	46·3	90·9	110·9	23·1	61·7	739·4	301·0	493·5	613·7	330·0	454·6
70–74	29·1	37·3	34·2	43·8	46·7	45·6	554·7	355·2	433·1	729·9	345·9	495·8
75 +	46·3	—	14·5	46·3	—	14·5	463·2	253·8	319·4	625·3	285·5	392·0

TABLE III.—*Comparison of Age Adjusted Death Rates for Cancers of the Liver (List No. 155–156) and Pancreas (List No. 157) and for All Cancers (List No. 140–205)*

Country	Liver			All cancer			Pancreas		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
Singapore, 1957	17·62	5·78	11·77	112·8	79·1	90·8	1·29	0·46	0·69
Singapore, 1957–58	16·98	5·22	11·21	121·2	73·5	94·9	1·41	0·71	1·00
England and Wales, 1956–57	3·38	3·09	—	170·9	113·4	—	6·36	4·01	—
Japan, 1956–57	15·20	9·36	—	127·6	94·0	—	2·18	1·49	—
Australia, 1956–57	3·57	3·63	—	130·9	106·4	—	6·14	3·51	—

TABLE IV.—*Comparison of Number of Cases Observed in the Singapore Population with the Number Expected for the Populations of England and Wales, Japan and Australia. (For details see Text)*

	Liver		All cancer		Pancreas	
	Male	Female	Male	Female	Male	Female
England and Wales	10·4*	10·2	574·0*	420·4*	19·5*	12·6*
Japan	52·5*	32·4	452·3	361·8*	8·8	5·7
Australia	11·8*	11·9	415·7*	365·5*	18·9*	11·2*
Singapore	78·0	20·0	498·0	299·0	5·0	2·5

the age specific death rates by 5-year group, for these three countries for 1956–57, as given by Segi (1960) were linked with the Singapore 1957 census population. On the assumption that cancer is a rare event occurring in a large population, and that the numbers observed will follow a Poisson distribution, significant differences, at a probability level of 0·005 (single tail) are indicated by an asterisk (*). The limits of the expectation are taken from Fisher and Yates (1957).

Bearing in mind the limitations of the basic data, cancer of the liver (List No. 155–156) is significantly commoner in Singapore males than in the male populace of England and Wales, Japan, and Australia, whereas in the female there is no such difference. Cancer (List No. 140–205) is more frequent in the male of England and Wales. Pancreatic tumours are more prevalent in both sexes in England and Wales, and Australia, than in Singapore.

Comparison with earlier years is always of interest, despite differences in the presentation and grouping of data.

In 1907–12 a series of 121 post mortems on cancer patients was carried out at Tan Tock Seng's Hospital, Singapore. This institution was at that time a free hospital for male adult paupers, principally of Chinese race. 25·6 per cent of these tumours were in the liver (Hoffman, 1915).

Hoffman (1935) analysed mortality from 1028 various cancers in Singapore for the years 1926–31. This study is particularly valuable, despite the broad groupings of the various tumours, as a racial breakdown was given. Using these figures, and the population data of the April 1931 census, when in a population of 555,745 individuals 75 per cent were Chinese, 12 per cent were Malaysians, 9 per cent were Indians or Pakistanis, Europeans and Eurasians each 1 per cent and Others 2 per cent, the mean annual rates for cancers of the stomach and liver per 100,000 persons per annum were respectively 23·1, 8·9, 13·0, 12·4, 29·0, and 24·1, i.e., a mean incidence of 20·4. In 1948–58 the incidence of these malig-

pancies was 15·5 per 100,000 population, reflecting, not a decrease in incidence, but a vast increase in the number of children on the Island.

At this time the male/female ratio for these tumours was 4·4 to 1 and carcinomata of the liver and stomach accounted for 55·4 per cent of all malignant tumours, whereas in 1948–58 they comprised 34·4 per cent.

Tull (1932) found 134 cases of primary liver cancer in a total of 17,664 autopsies carried out at Tan Tock Seng Hospital, Singapore, an incidence of 0·76 per cent. This figure is rather lower than might be expected in a free hospital for male adult paupers. The 90 per cent autopsy rate and the higher death rate at this time from tropical disease possibly accounts for this. Using data supplied by Tull, Bonne (1937) noted that in Singapore, the liver was the primary site in 36 per cent of 128 post mortems on cancer cases.

In Malaya (1925–31) Hoffman (1935) found that 46·8 per cent of the 1493 deaths ascribed to malignancy were caused either by gastric or by hepatic neoplasms. In the same septennium these tumours accounted for 24·8 per cent of 1780 cancer cases treated in hospital.

In view of the paucity of post mortems on Malays, the biopsy data collated by Marsden (1958) from 4650 malignant tumours submitted for opinion to the Institute for Medical Research, Kuala Lumpur, are of significance. 7·5 per cent of all malignant neoplasms in Chinese, 3·0 per cent in Indians, and 2·7 per cent in Malays, arose in the liver (List No. 155), this despite the reluctance of Malays to seek hospital treatment, let alone submit to biopsy. Marsden (1958) calculates the apparent incidence rates for Chinese and Indians to be 6·2 and 2·2 per 100,000 living per annum respectively (Singapore mean, for all races, for List No. 155–156, 6·3 per 100,000) and remarks that the incidence in Malays is probably as high as in Chinese.

In 1948–57 there were 2032 admissions to Malayan hospitals with liver cancers (List No. 155–156), 46 per cent dying from the disease, a morbidity of 2·5 per 100,000 per annum (Singapore : 6·4 per 100,000 per annum).

The Registrar General (Malaya) does not give a detailed breakdown of deaths in his reports, rightly considering that to do so would be misleading as only 20 per cent of deaths are certified by medical practitioners.

There seems little doubt that liver cancer is very frequent in South-East Asia.

Vedder (1927) in a post mortem series on 157 Filipino cancer cases found that 39 per cent of tumours arose in the liver or gall bladder. Kouwenaar (1951) found in Java, on analysis of the post mortem records of a group of estate hospitals, that of 120 Chinese males who died from some form of malignant disease, in 31 per cent the primary tumour was hepatic. In 59 Javanese males the proportion was much higher, 78 per cent. These figures are astonishingly high, and are even more remarkable when one realises that these figures represent respectively 2·8 and 3·9 per cent of all the necropsies performed. While it is likely that this population was a selected one, Kouwenaar (1951) does not state its composition.

Nguyen-Van-Ai (1958) has described the malignant tumours in South Vietnamese based on biopsy material. Of 1303 malignant neoplasms only 6 per cent were from the digestive tract, and but 0·8 per cent arose in the liver. In Thailand, again in biopsies, 5 per cent of 350 malignant tumours were considered hepatic in origin (Vellios, Goonchorn, and Suvanatemiya, 1953).

Cooray (1954) found 4 liver cell tumours, 2 tumours of the gall bladder and bile ducts, and 2 tumours of the pancreas in 57 post mortems on cases of malignant

disease at Colombo, Ceylon. In 1815 malignant biopsies corresponding figures were 9, 1 and 2. Cooray (1951) points out that too much reliance cannot be placed on these figures as post mortems are few in number.

Mitra and Gupta (1960) have attempted to estimate the overall incidence of cancer in India by applying death registration figures for the City of Calcutta to the sub-continent. In the Calcutta corporation area no corpse can be disposed of without a death certificate from a competent medical officer, although this often means a certificate based merely on the reported history of a case after death has taken place. In a total of 1645 deaths from malignancy, 276; or 16·7 per cent, were due to liver tumours. The male/female ratio was 1·4 : 1. The peak incidence, 36 per cent of cases, was in the age group 60 and over. If these figures hold good for the whole of India, as the authors suggest they may, then each year there must be, in round figures, 30,000 new cases of liver cancer. Based on an estimated population of 380 million, of an age structure not unlike that in Singapore, the crude mortality rate for these tumours must be in the order of 7 to 8 per 100,000, a value not far removed from the Singapore estimate of 6·3 per 100,000.

Despite these vast numbers, in 1957 Thompson, Hackley, and McGinnis, in a review of the world literature were able to collect only 2205 cases, a reflection of the vast pool of material still to be tapped in the Orient.

A perusal of the above data forces one to the conclusion that in South-East Asia there is but one territory where figures of morbidity and mortality from malignant disease can be usefully compared with those from elsewhere, i.e., Singapore.

In the West, hepatic carcinomata comprise about 0·12 per cent of all post mortems and about 1·2 per cent of all carcinomata (Büngeler and Eder, 1960). Extremely high figures have been reported from isolated centres such as Tobolsk in Soviet Siberia where cholangiocarcinomata, associated with opistorchiasis, comprised 3 per cent of all autopsies (Kraft, 1958).

As the majority of tumours in this paper were in Chinese, data from China are of great interest. From January 1950 to June 1957, 21,706 post mortems were carried out in 38 medical colleges. One thousand nine hundred and seventy-nine malignancies were encountered (9·1 per cent), and of these 262 (13·2 per cent) were primary liver tumours, i.e., 1·2 per cent of all necropsies. The incidence in hospital admissions varied from 0·07 to 0·3 per cent. Most of these liver tumours were seen in persons between the ages of 31 to 50, the mean age being 40 (Yeh, 1959).

Yeh and Cowdry (1954) report that in 1025 autopsies carried out in Formosa (Taiwan) there were 140 instances of malignant disease (13·7 per cent) of which 28 (20 per cent) arose in the liver.

The biological behaviour of liver tumours has attracted some attention. Shanmugaratnam (1956) states that cholangiocarcinoma seems to be equally prevalent in both East and West, and it is only the hepatocellular tumours which are the more numerous in the Orient. Reviewing the literature he finds that bile duct tumours are more frequent in females, unlike the liver cell variety, and that extra-hepatic metastases are frequent and widespread.

In addition to the differentiation between hepatocellular and cholangiocellular tumours, various attempts have been made to further classify the tumours arising in the parenchymal liver cells. Liang and Tung (1959) divided their 38 cases into two major types, the massive and the nodular, these occurring in their

material with roughly equal frequency. Almost all of the massive tumours were situated in the right lobe, with or without smaller satellite nodules. Tumour thrombi in the portal vein were rarely seen, whereas lung metastases were common. This type of tumour was usually found with a Laennec type of cirrhosis. Death was due either to cachexia or to intra-abdominal rupture of the main tumour mass.

The nodular tumours they further separated into mononodular and multinodular. Four of five of their cases of mononodular carcinomata were situated along the Sereje-Cantlie line, i.e. from gall bladder to inferior vena cava. Generally livers with nodular carcinoma were lighter in weight than the massive type. The portal veins were frequently involved whereas pulmonary secondaries were relatively rare. Mononodular tumours were associated with post-necrotic scarring, the multinodular with Laennec's cirrhosis. Death usually followed haemorrhage from either ruptured oesophageal varices or from intra-abdominal rupture of the tumour.

Histological differences were tentatively suggested : mitotic figures and tumour giant cells were more frequent in massive tumours, stromal fibrosis being commoner in the nodular type.

The authors do not give figures for some of their observations, using terms like " frequent, rare, relatively rare, etc." in comparison. Analysis of the given figures reveals one statistically significant difference ($P < 0{\cdot}01$) : an increased prevalence of lung metastases in the massive tumours.

Using these criteria, an attempt was made to classify the present material. This was not easy and more reliance was placed on the photographs accompanying Liang and Tung's (1959) communication, than on the textual description. Division into mononodular and multinodular types was not possible. With these reservations 60 per cent of tumours fell into the massive category, 33 per cent into the nodular, and 7 per cent could not be classified.

Ruptured oesophageal varices were found in 5 per cent of massive tumours, and in 17 per cent of the nodular. Corresponding figures for intra-abdominal haemorrhage following rupture of the tumour were 20 per cent and 35 per cent. The differences are significant ($P < 0{\cdot}05$). There were fewer lung metastases, and more varices, with more frequent invasion of the portal vein, in the nodular than in the massive, but the differences are not significant. On average, the nodular livers were 170 g. lighter.

The figures for this series are thus in broad consonance with those of Liang and Tung (1959) from Canton, although the division is by no means clear-cut.

Shanmugaratnam (1956) found a disparity between the immigrant Chinese from South China and the indigenous Chinese of Singapore, who are of the same ethnic origin, in their susceptibility to develop liver cell carcinoma. Now that new immigration from China has ceased, and that fewer Singapore Chinese return to China for extended holidays, if his hypothesis that there is a liver cancer inducing environmental factor present in China, and absent in Singapore, is correct, then his analyses over the next thirty years, as the China-born Chinese die off, will be of great interest.

There is some evidence that the incidence of liver cell cancer is higher in South China than in the North (Liu, 1953), a difference which has been ascribed to Clonorchiasis. Chlonorchiasis and schistosomiasis were rare in the present series : neither parasite is found in Malaya or Singapore. Yeh (1959) quoting Lin (1958)

states that of 537 cases of primary liver cancer reported in Chinese literature in the past 10 years only 10 showed a concomitant schistosomal infection, and that in these there appeared to be no relationship between the ova and the growth. The significance of *Clonorchis sinesis* infection has been extensively investigated by Hou (1956).

SUMMARY

The morbid anatomy of the 178 primary malignant neoplasms of the liver seen at autopsy in Singapore in the period 1948–58 inclusive is presented.

The age and sex incidence are tabulated. The peak incidence is in the fifth decade, more than half the deaths taking place before the age of 50.

In the post mortem room cancers of the liver, stomach, and lung are seen in virtually equal numbers, but in the returns of the Registrar General (Singapore) the respective ratios of incidence are 1 : 2 : 1. The crude mortality rate, based on registered deaths, from liver cancer (List No. 155–156) is 6·3 per 100,000 living per annum.

Age-specific death rates for cancer (all sites) and for cancer of the liver are given for 1957 and 1957–58.

The incidence of cancer (all sites), and of cancer of the liver and pancreas is compared with that of England and Wales, Japan, and Australia, by derivation of age-adjusted death rates, and by calculation of expected numbers.

Cancer of the liver is seen to be significantly more prevalent in the Singapore male, while cancer (all sites) is significantly commoner in the male of England and Wales.

Tumours of the pancreas and peritoneum are briefly mentioned. In both sexes pancreatic tumours are shown to be significantly less common than in England and Wales, and Australia.

A division of the liver tumours into massive and nodular types, as proposed by Liang and Tung (1959), is attempted, and some of the differences in biological behaviour noted by these writers are tentatively confirmed.

A portion of the relevant literature from South-East Asia, India, and China is discussed, and the importance of Singapore as a centre for epidemiological research in this part of the Orient is emphasized.

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HAEMATOLOGICAL FINDINGS IN AFRICAN CHILDREN IN UGANDA WITH MALIGNANT LYMPHOMA

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In recent years it has been found that lymphosarcoma is one of the commonest tumours of Africans in Equatorial Africa and that it has a very curious predilection to occur multicentrically in the jaw bones, particularly but not exclusively in African children. Clinical descriptions of these jaw cases have been given by Burkitt (1958), descriptions of the pathological and radiological aspects by Davies and Davies (1960) and by Davies (1959). Meanwhile O'Conor and Davies (1960) have shown that lymphosarcoma or malignant lymphoma is responsible for almost one-half of all malignant growths in African children. Detailed studies of the geographical distribution of this tumour have been made by Burkitt and O'Conor (1961) and of the histopathology and cytology by O'Conor (1961).

It has been shown that in African children about half the cases of lymphoma occur with involvement of the jaw, in the remainder as in the cases where the jaw is affected, there are tumour deposits in many organs; infrequently bones other than the jaw show tumour deposits. The tumour is highly malignant, growth is extremely rapid and death usually occurs in a few weeks.

O'Conor and Davies (1960) have conversely drawn attention to the comparative infrequency with which leukaemia appeared in their series of African children, and as lymphoma in frequency occupies the position usually held by leukaemia in America and Western European countries, the question arose as to whether this might be a "biological variant" in the response of reticulum or haemopoietic tissue to some unknown agents. In order to establish whether there was any reflection in the peripheral blood or marrow of the widespread lymphomatous process, a haematological survey was undertaken of 32 histologically proven cases of this malignant lymphoma in African children.

MATERIALS

The WBC cases were drawn from those cases with a histologically proven diagnosis registered with the Kampala cancer survey 1951–60 including only those cases in which a full white cell count had been performed. The myelograms were done on 5 cases initially diagnosed in the first half of 1960. Since as yet no method of diagnosing this disease exists before the appearance of a macroscopic tumour, all cases investigated were perforce seen at a relatively advanced stage. But apart from this they covered all stages from a child with one small tumour seen within a few weeks of clinical onset to those with many deposits, some with gross ulceration.

METHODS

White cell counts were performed according to the method of Dacie (1956) using a Neubauer chamber. The mean of counts on two fillings of the chamber was taken as the value. Differential white cell counts were done on thin films stained by May-Grunwald Giemsa stain. Results were based on a count of 200 cells.

Bone marrow was obtained from the iliac crest, less than 0·5 ml. of marrow being taken : after aspiration of surplus blood films made of the fragments were then stained by May-Grunwald Giemsa stain. The myelogram was then obtained from a count of 500 cells.

RESULTS

Circulating white cells. African lymphoma children, 32 cases

The mean age of the children was 6·6 years, with an age range of 2–14 years. The sex ratio was male 23 : female 9.

TABLE I.—*Circulating White Cells/cu./mm. Lymphoma Children*

	Mean	Range	Values for normal American children (Albritton, 1952)
Total count	8,003	4,600–13,300	8,600
Neurophils	3,774	2,000–7,200	4,200
Eosinophils	689	100–3,800	200
Basophils	2·2	0–70	40
Lymphocytes	3,381	1,100–5,300	3,800
Monocytes	387	70–1,200	400

TABLE II.—*Bone Marrow Constituents*

	Lymphoma children		Adult Africans (Van den Bergh & Blitstein, 1945)	
	Mean (per cent)	Range (per cent)	Mean (per cent)	Range (per cent)
Eosinophils	11	8–15	6	1·8–10·8
Myeloblasts	1	0–2	2	0·6–3·2
Promyeloblasts	2	1·2·5	3·6	0·4–8
Myelocytes	17	14·4–21	19·3	5·2–38
Metamyelocytes	22	7–30	28·4	8·4–44·2
Segmented neutrophils	6	0–9	9·6	1·2–22
Lymphocytes	6	0–11	29·3	12·6–47
Monocytes	0·8	0–2	1·2	0·4–2·6
Plasma cells	3	2·5·5	3·1	0·2–10·8
Total erythroblasts	30	16–45·5	25	18·2–47

DISCUSSION

It can be seen from the tables that there is no evidence of any leukaemic condition present in these children either as judged from the circulating white blood cells or from the myelogram. Thus the malignant lymphoma of African children is not associated with any changes suggestive of a close relationship to a leukaemic condition. This is in agreement with the clinical evidence.

It is more difficult to be sure that there are no slight deviations from the normal blood and marrow picture of African children in the lymphoma cases as a little difficulty has been encountered in obtaining normal values for a similar group of African children. Albritton (1952) has given the normal values for leucocytes in American children in the age range 2–14 and these are shown in the third column of Table I. No exactly comparable data seems to exist for African children. A number of observers (Blitstein, 1950; Linhard, 1956; Moore, 1958) have noted that in the African adult there is a relative increase in the number of circulating lymphocytes (and a moderate increase in the eosinophils) with a relative decrease in the number of neutrophils together with a decrease in the total white blood count. These seem to be features seen in African children (Blitstein, 1950). If therefore due allowance is made for such a racial factor, then there is no significant difference in the circulating white cells between normal African children and those with lymphoma.

Similar difficulties occur in interpreting the finer differences in the myelogram. The normal values vary greatly according to the method used to obtain marrow, the method of making films and the terminology used by the observer. The myelograms from a group of clinical normal African adults (van den Bergh and Blitstein, 1945) is given in Table II together with the values obtained in the lymphoma cases. The greatest variation is seen in the lymphocytes, where, contrary to expectations, there appears to be a significant decrease in values for the lymphoma cases. But though the lymphocyte levels found in this lymphoma series differs from those found by Van den Bergh and Blitstein (1945) in African adults they are in accord with levels quoted for normal children elsewhere (Albritton's value 9·8 per cent, range of 21 authors' means 2·7 to 24 per cent). In the absence of strictly comparable series of normal African children no firm conclusion can be drawn.

Van den Bergh and Blitstein (1945) found increased marrow plasmacyte levels in the normal Africans in the Congo and related this to disorders of protein metabolism and chronic marrow irritation. In this lymphoma series the marrow plasmacyte levels are comparable with those of Blitstein (1950) but above the values quoted by Albritton (1952; 0·1–1·5 per cent, Mean 0·6 per cent). The significance of this is uncertain but it has been suggested (Dunn, 1960, personal communication) that this African lymphoma may be a disorder of plasmacytoid cells in which case this raised plasma cell level may be of importance.

While an occasional prolymphocyte and an occasional Türk cell are seen in circulating blood in these cases of proven lymphoma, they were seen just as frequently in a control series of normal African children. This experience does not lead us to think that the finding of prolymphocytes in the circulating blood with a slight increase in the total lymphocyte count would enable us to predict the onset of lymphoma before the development of macroscopic tumours. This possibility has been raised by the studies by Bendixen (1958, 1959) of lymphocytic bovine leukosis in Denmark. Recent work in this field has been summarised by Steere (1959). This disease of young cattle has a geographically localised distribution depending on the movements of presumed infected animals. The infective agent is believed to be a filterable virus, there is a long incubation period, which explains the peculiar epidemiology of the disease, and there is ultimate involvement of the entire reticulo-endothelial system. This bovine disease has a marked superficial resemblance to the lymphomas seen in African children with respect to

tumour morphology, cellular pattern and the distribution of " secondaries ". With increasing knowledge of this disease Bendixen was able to diagnose it by the method mentioned before the development of macroscopic tumours. Our attempt to show such changes in African children was unsuccessful but further investigations are necessary.

Since the commencement of these observations a number of children with lymphomas have been treated with Methotrexate (4-amino-N^{10} methylpteroylglutamic acid). All the children treated showed a marked increase in the level of the circulating lymphocytes. The significance of this is unknown and is being further studied.

SUMMARY

1. Circulating leucocyte and marrow cell counts were performed on a series of African children with lymphoma and compared with the most relevant published normal data.

2. No significant diagnostic variation of circulating leucocyte levels or morphology have been noted.

3. No significant variation from the expected myelogram pattern has been observed.

4. There is no evidence of a high level of circulating prolymphocytes and it is doubtful if a prognostic test can be devised.

5. Lymphoma in African children shows no blood or marrow features which could be considered as " leukaemic ".

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THYROID FUNCTION IN RELATION TO MAMMARY CANCER

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THE direct or indirect influence of certain hormones, e.g. stilboestrol and testosterone, on the aetiology of malignancy has been well recognised for many years and has formed the basis of their extensive use in the management of this disease, particularly in attempting its control in advanced stages. Some of the cancers of breast and of prostate are good examples of tumours which are hormone dependent. But the role that the thyroid hormone plays is still uncertain. Beatson was the first, in 1896, to treat advanced carcinoma of breast with thyroid extract in addition to oophorectomy. Only in the last decade or so the result of a good deal of experimental work and clinical observation has been to suggest that thyroid secretion plays an important part in the evolution of cancer, particularly of the genitalia and the breast. Loeser (1954) states that the incidence of female breast carcinoma is far lower in allergic and hyperthyroid conditions than in hypothyroid women or after thyroidectomy. In his view, it is the low histamine content of the cells of hypothyroid women which increases the tendency to cancer formation. Spencer (1954), discussing iodine availability in cancer incidence, doubts whether a low metabolic rate or insufficiency of thyroid substance can be considered as a primary cause of cancer. He suggests, rather more vaguely, "that thyroid function (or dysfunction) may be associated with the susceptibility or immunity to cancer". Sommers (1955), after examining autopsies of breast cancer cases and of about an equal number of adult women without cancer, found hyperplastic modification in one or more endocrine glands and their target organs. A noticeable exception was the thyroid gland, in which atrophy occurred more commonly with than without breast cancer. The thyroid atrophy was independent of the body weight.

Dargent and Mayer (1955), surveying 71 cases of cancer of various sites, have attempted to demonstrate that thyroid secretion has a definite influence on the evolution of cancer, and warn that surgical treatment of goitre in a case of established malignancy should not be undertaken lightly. Dessaive (1956) in both his experimental work and clinical observations comes to a similar conclusion. From his study of 13,261 cancers seen in Liége it appears that simple thyroid conditions, particularly goitre, constitute a favourable element in the formation of cancer. The effect was found in women much more often than in men, and was especially noticeable in breast carcinoma. He also found that cancers which develop against a background of thyroid deficiency have a worse prognosis, and recommends full investigation before undertaking surgical or other treatment for simple thyroid disorder, in order to exclude the presence of a cancer which at that time may be

quiescent. He postulates the theory that thyroid deficiency creates changes in the hypophysis which in their turn cause conditions favourable to cancer evolution. According to him the hyperthyroid state produces a barrier to cancer formation. In support of this hypothesis he mentions that cancer mortality is very high in regions where goitre is endemic.

Ellerker (1956) in his survey of 157 women with breast carcinoma found that 7·8 per cent had thyroid anomalies and concurs with other authors that hypothyroidism may be a factor in the formation and spread of cancer. Following this line of thought Loeser (1958) advises thyroid administration after surgical or X-ray treatment of the primary tumour which in his opinion can prevent recurrence " for a long time, possibly for most of the patient's life ". He treated 56 women with carcinoma of breast or genitalia in this way and claims that recurrence has been prevented " in the great majority for five years and longer ". He has lately been injecting solution of thyroid in and around the tumour, as well as giving thyroid combined with histidine (Loeser, 1960, personal communication). Recently, Edelstyn, Lyons, and Welbourn (1958) have demonstrated a relationship between thyroid function and metastatic spread in cancer of the breast: function was significantly lower in patients with bloodborne metastases than in those with only local disease.

PRESENT INVESTIGATION

In pursuit of the same line of thought a survey of a group of female patients suffering from mammary carcinoma was begun in 1956 in Coventry. Thyroid function tests were carried out either before or at the initial stage of treatment in order to discover any dysfunction of the thyroid gland.

One hundred and nineteen women with various types of breast carcinoma were investigated by radioactive iodine (^{131}I), and 98 of these had their blood cholesterol estimated also. In all these cases the neoplasm was still localised to the breast tissue or to regional glandular areas and had no distant metastases. One hundred and fifteen had simple or radical mastectomy followed by radiotherapy. Only four patients were found not suitable for surgery and were treated by radiotherapy alone, but even so it was possible to use a high radical dose. The mean age of these patients was approximately 54 years (Table III). The isotope tracer doses were given either before commencing or at an early stage of radiotherapy, to avoid any alteration in thyroid function due to the radiation itself when treating the supraclavicular region. Absorption of iodine in the thyroid gland and excretion of the isotope in the urine were both measured. Initially the uptake tests were made using a stand in which the whole of the gland area was viewed by a G 10 Pb counter through an aperture in a 1 in. lead screen. Later measurements were done with a " collar " arrangement of screened counters around the neck of the patient. In all cases the uptake in the neck was compared with a standard dose contained in a 25 ml. perspex model of a thyroid gland embedded in a wax model of an average neck.

The uptake measurements were originally done at 1, 2, 4, and 24 hours but later the measurement at 1 hour was dropped. For the excretion test the urine was collected during the periods of 0–2 hours, 2–4 hours, 4–6 hours, 6–8 hours, 8–12 hours, 12–24 hours and for low uptake patients 24–48 hours. The criteria for the results were the same as those in general use for thyroid investigations. These criteria are summarised in Table I. Cholesterol was estimated by the method of

Zak *et al.* (1954) and the present figures were compared with the normal range by age found by Keys *et al.* (1950).

TABLE I.—*Criteria Adopted in Assessment of Thyroid Function by Gland Uptake and Urinary Excretion of* ^{131}I

Gland Uptake (% of ingested dose)

Period	Normal	Hyperthyroid	Hypothyroid
1st hour	7–19%	20–50%	4–11%
2nd hour	<25%	>25%	—
4th hour	<40%	>40%	—
24th hour	13–55%	46–96%	1–10%

Excretion (Urine) (% of ingested dose)

Period	Normal	Hyperthyroid	Hypothyroid
8–12 hours	3–10%	0–3%	—
		severe mild	
6–24 hours	12–38%	0–4 ; 4–12%	—
8–24 hours	7–20%	0–3%	16–34%
24–48 hours	0–10%	0–4%	8–23%
8–48 hours	9–25%	—	36–51%
0–24/48 hours	37–70%	4–47%	60–91%

All except three cases were staged clinically according to the Manchester four-stage system : in the three cases the clinical information was inadequate to permit allocation to a stage, and they appear therefore as N/K. Cases were divided pathologically into the three groups :

Early, where the excised glands were found histologically to be non-malignant.
Late, where the lymph nodes were found to be involved.
N/K, where there was no microscopical examination of the glands.

RESULTS

Of the 98 patients who had serum cholesterol estimations, 18 were considered to have either high or possibly high readings and 7 cases had either low or possibly low figures. The range of serum cholesterol readings was from 128 to 440, the mean being 259·11 mg. per cent and the standard deviation 58·13. There was a slight, though not significant, positive correlation between serum cholesterol level and age. This accords with the trend with age shown by Keys *et al.* (1950), particularly for the range of ages included in the present study.

Table II shows the mean, standard deviation, range, and number of cases for each of two ^{131}I uptake (A, B) and two excretion (C, D) figures. The correlation coefficient between A and B (0·803) is so high that it is here sufficient to use one of these readings only, despite the difference in mean. Much the same is true of the readings C and D for urinary excretion (correlation coefficient =0·772). Only B therefore was subsequently used for uptake, and C for excretion.

There was a high degree of negative correlation between B and C, as of course would be expected since an increased uptake implies a reduced excretion. Between

cholesterol and B or C the correlation was so small as to indicate no sensible relationship between these readings.

TABLE II.—*Distribution Within Experimental Series of Certain Uptake and Excretion Values of* ^{131}I

	^{131}I Uptake		^{131}I Excretion	
	A	B	C	D
Mean	17·85	38·78	20·0	19·15
S.D.	6·31	11·97	6·70	8·07
Range	2–48	3–65	3–36	2–45
(n)	(118)	(119)	(118)	(86)

A = % ^{131}I Uptake (2nd hour);
B = % ^{131}I Uptake (24th hour);
C = % ^{131}I Excretion (6–24 hours);
D = % ^{131}I Excretion (8–48 hours).

The mean values of B (uptake 24th hour), C (excretion 6–24 hours), age, and cholesterol are given in Table III for each clinical stage, and in Table IV for each pathological stage, together with the numbers of cases in each group. Here again there is no very noticeable trend in any of the values shown.

TABLE III.—*Mean Values by Clinical Stage of :—*^{131}I *Uptake—*^{131}I *Excretion—Age—Serum Cholesterol*

	Clinical stage				
	I	II	III	IV	N/K
	(38)	(43)	(26)	(9)	(3)
B	39·1	38·0	37·8	44·2	38·0
C	20·8	20·0	19·3	18·3	20·0
Age	53·8	53·1	55·8	58·0	55·3
	(33)	(39)	(17)	(6)	(3)
Cholesterol (mg. %)	249·6	265·2	259·5	238·8	322·7

B = % ^{131}I Uptake (24th hour).
C = % ^{131}I Excretion (6–24 hour).
Figures in brackets denote the number of cases in each group.

TABLE IV.—*Mean Values by Pathological Stage of :—*^{131}I *Uptake—*^{131}I *Excretion—Age—Serum Cholesterol*

	Pathological stage		
	Early	Late	N/K
	(24)	(57)	(38)
B	38·4	39·3	38·0
C	20·3	19·2	21·2
Age	50·7	54·2	57·0
	(23)	(43)	(32)
Cholesterol (mg. %)	247·9	257·9	268·8

B = % ^{131}I Uptake (24th hour).
C = % ^{131}I Excretion (6–24 hour).
Figures in brackets denote the number of cases in each group.

In the case of gland uptake, our experience (24th hour) corresponds very closely with that of Edelstyn *et al.* (1958) for their group with local disease only. Their mean value (37·6 per cent) and ours (39·1 per cent) are virtually indistinguishable, and the standard deviations (9·77 and 11·78) also accord well together. Our range (3–65) is more extensive than theirs (25–65) as might be expected in a sample nearly 5 times as large (119 against 26); only 4 of our 119, however, are below 20 per cent.

TABLE V.—*Distribution of T-index* (*Fraser* et al., 1953)

Range		No.	%
Very Low	(≤2·1)	4	5
Low	(≤2·8)	8	10
NORMAL	(≤12·8)	68	82
Probably high	(≤17·4)	1	1
High	(>17·4)	2	2
		83*	100

* For various reasons it was not possible to obtain a T-index for all our patients.

For urinary excretion we have calculated the T-index of Fraser *et al.* (1953) on 83 of our patients. Table V shows their distribution in terms of the five groupings used by Fraser. We have 82 per cent within his Normal range, 3 per cent above it, and 15 per cent below. Of the latter, 4 (5 per cent) only are within his Very Low or myxoedematous group. Before the T-index was computed, these four patients were classified clinically. In two, myxoedema was mentioned, and the other two were described as " low normals ". Of the 8 in the next group, three were described as possibly myxoedematous, and the remaining five normal. The three classified above Normal in Fraser's grouping were all described as " hyper ", though two of them were only mildly so. Thus our results show a slight bias towards hypofunction in their distribution, although the mean value works out at 6·3, due to the inclusion of the High group, one of which had a T-value of 90.

From the diagram published in their paper, Edelstyn *et al.* (1958) show an even greater bias towards hypofunction, for their group with local disease only—the group most nearly comparable with our own. Of their 23 cases, 17 are in the Normal group, 4 in the Low, and 2 in the Very Low, making 26 per cent below Fraser's lower extremity of the Normal group. Taken in conjunction with our more equivocal findings, it is perhaps suggestive of a reduction in thyroid function, manifested in patients with breast cancer, even when confined to local spread only. However, neither Edelstyn *et al.* nor we, for similar reasons, have studied a control group of individuals without cancer.

DISCUSSION

In our series, where the growth was still localised to breast tissue or to regional glands, without distant metastases, there is no apparent association with thyroid dysfunction. This is in accord with the main findings of Edelstyn *et al.* (1958) in cases of local disease. Indeed, we might on this evidence hazard the observation that thyroid dysfunction plays little part in the primary aetiology of mammary cancer, apart from the slight bias towards hypofunction demonstrated above. The interest now focusses on the role of the thyroid in metastatic spread of the

disease, and on whether the association, reported for instance by Edelstyn *et al.* (1958), between thyroid dysfunction and blood borne metastases is a consequential or a concomitant one. Does a reduced thyroid output in some way facilitate metastatic growth, or does the presence of an active growth depress the thyroid, either directly or through the pituitary ? Alternatively both manifestations may be due to some third factor not yet clearly identified. The importance, both therapeutic and theoretical, of elucidating this relationship demands a programme of detailed observation and appraisal. Tests of thyroid function should be made at the outset, before treatment is given—certainly before radiotherapy which might well disturb the pattern. Thereafter a careful follow-through study is necessary to determine the subsequent fate in terms both of metastatic spread and of thyroid function. Only by such prospective and continuing studies can we hope to obtain an answer to the fundamental question of " which comes first? ". The material we present here cannot yet attempt to provide an answer : it is only in a very early stage. We believe however that the basic question is of sufficient importance to justify such a preliminary communication, if only to stimulate the collection of similar data on a larger scale. Our own cases have not yet been followed for more than three to four years. We shall of course keep a careful watch on them in order to assess progress and survival according to their thyroid function.

SUMMARY

Thyroid function was determined by means of radioactive iodine uptake and urinary excretion in a series of cases of breast cancer localised to the breast or regional glands. Ninety-eight of the series of 119 had also estimations of blood cholesterol. Although there appeared to be a slight bias towards hypothyroid function, no evidence of statistically significant thyroid dysfunction was obtained. All these cases are being followed to determine whether an association exists between thyroid dysfunction and metastatic spread.

We wish to thank Mr. J. D. F. Williams, principal physicist at the Coventry and Warwickshire Hospital, for undertaking the iodine studies ; Mr. R. W. Richardson, senior biochemist of the same Hospital, who was responsible for the cholesterol estimations ; and Mrs. Valerie Evans for much secretarial and computational assistance.

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RELATIONSHIP OF DIFFERENT HISTOLOGICAL LUNG TUMOUR GROUPS TO TOBACCO SMOKING

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THE author in a series of papers (1954*a*, *b*, *c* and *d*, 1955, 1959) has accumulated evidence to show that the different histological types of primary epithelial lung tumours have different relationships to the sex, domicile, occupation and smoking habits of the patients.

The lung tumours have, accordingly, been separated into two main groups, and the ratio Group I : Group II tumours has, especially in males, been shown to be an important means of measuring such differences.

In the paper of 1955 this ratio was also applied to the study of the relationship between the occurrence of lung tumour types and tobacco—especially cigarette—smoking.

An analysis of the material then at hand, consisting of 258 male cases, strongly pointed in the direction that no such relationship exists as regards the Group II tumours, whereas the Group I tumours rapidly increases with the amounts of tobacco smoked.

A few years later Doll, Hill and Kreyberg (1957) confirmed these findings in British material. From the latter paper Fig. 1 is reproduced, and here the rate per 100,000 for the two groups at different smoking levels as well as the risk to develop lung cancer of the two groups related to the risk in non-smokers are given.

Whereas the Group II tumours show no certain increase with increased tobacco smoking the relationship in the Group I tumours is very marked and follows a straight line.

The author's Norwegian material has in recent years increased to a total of 600 cases, of these 522 males, and this enlarged material has now been examined as to the Ratio Group I : Group II tumours at different smoking levels. The result is given in Table I.

TABLE I

Group I : Group II

Norway

Smokers males

Grammes smoked per day	Number	Ratio
0	4:13	0·3:1
—4	5:11	0·5:1
—9	69:31	2·2:1
—14	147:39	3·8:1
—19	67:12	5·6:1
—29	75:9	8·3:1
30+	36:4	9·0:1

A most remarkable and regular increase is found, and using the ratio for non-smokers as a basis the risk to develop Group I tumours for each smoking level studied has been plotted in Fig. 2.

A very striking similarity can easily be seen between the British and the Norwegian materials, and the order of magnitude of risk is nearly identical.

Also the Norwegian material shows a straight line correlation between frequency of Group I tumours and amounts of tobacco smoked, except in the lowest and highest levels. These deviations may be spurious because of the smaller

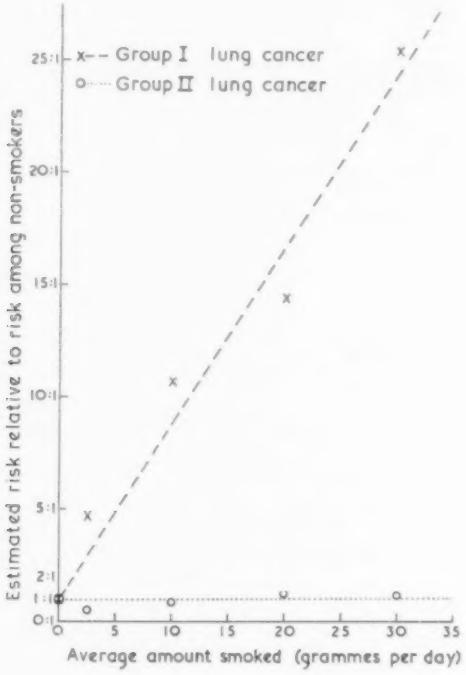

FIG. 1.

number of cases at these levels, but they may also reflect realities. This question can only be solved when a larger number of cases is available, or when a better knowledge of dose/tumour relationships has been obtained. Some care should be exerted as to comparison with animal experiments as such experiments usually are carried out on inbred strains whereas the human material is highly heterozygous.

In the British material the typing of the Group II cases and a sample of the Group I cases was done by one pathologist without knowledge of the sex or smoking habits of the patient or of the previous histological diagnosis. The remainder were typed by the hospital pathologist concerned before the question of a differen-

tial relationship with tobacco had been raised. After the typing had been completed in such a manner no revisions took place.

In the Norwegian material the typing took place consecutively on the arrival of the cases, but the review was not made until the 522 cases were collected. In this material also no revisions or adjustments were made.

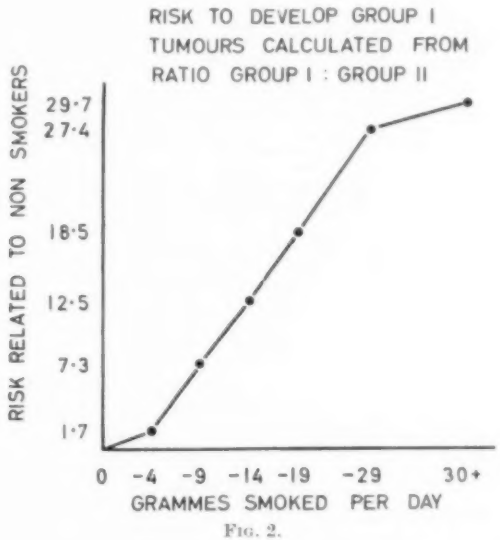

FIG. 2.

The results of the study of the two materials were obtained through the use of different methods. The British material was examined as to the *rate* occurrence and the Norwegian as to the *ratio* findings.

The similarity of the findings seems to permit only one reasonable conclusion, namely, that tobacco—especially cigarette—smoking is the essential causative factor involved in the development of Group I lung tumours, that is : epidermoid carcinomas and small cell anaplastic (" oat "-cell) carcinomas.

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POLYPLOIDY AND RADIOSENSITIVE BEHAVIOUR OF HUMAN MALIGNANT CELLS *IN VIVO*

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It is an established fact that malignant cells of the same type under the same radiation treatment react differently to the therapy. Goldfeder (1947) has shown that malignant cells of the same morphological type differ in radiosensitivity. Koller (1947) has also shown that the mean number of chromosome fragments per cell differs in cell samples taken from different regions of the same tumour after radiation treatment. In plant material, different authors have observed different types of correlation between polyploidy and radiosensitivity of normal cells. Smith (1946) and Bishop (1952) reported that the sensitivity of the polyploid cells is equal to that of diploids. Sax and Swanson (1941), Froier, Gustafsson and Tedin (1942), Sparrow (1957) and Oster (1958) are of the opinion that a high degree of radioresistance of normal cells is related to their high chromosome number. Puck (1960) demonstrated that *in vitro*, hyperploid and aneuploid normal human cells are more radioresistant than diploid cells. Révész and Norman (1960) have made a correlation study of chromosomal ploidy and radiosensitive behaviour of the ascites tumour cell population.

As the majority of malignant tissues of human patients are in solid form, so ultimate radiotherapeutic assessment should be made on solid tumour. Atkin, Richards and Ross (1959) and Richards and Atkin (1959) have made a study of some possible significance of radiotherapeutic effect and DNA content of different types of solid tumour arising from human uterus. They have suggested (Atkin *et al.*, 1959) that radioresistant cell strains are more often higher than diploid than near diploid. The present investigation was undertaken to study the relationship between the chromosomal population and radiosensitivity of epidermoid carcinoma cells of human cervix *in vivo*. The significance of this study is that here the nature of the biological material, i.e. the epidermoid carcinomatous condition, is kept constant.

MATERIAL AND METHOD

Twenty patients with histological evidence of epidermoid carcinoma in the cervix uteri (without any previous history of radiotherapy) of different ages, stages, grades and stromal proliferation (Tables II and III) were selected from the out-patients department of the Chittaranjan Cancer Hospital. Tissues were collected from the cervices of the above patients for chromosomal study. Chromosome counting was made on aceto-orcein squash preparation made according to the method suggested by Tjio and Levan (1954), the tissue being fixed in 1 : 3

aceto-alcohol after pre-treatment in hypotonic saline solution (Levan, 1956) for thirty to forty-five minutes. Chromosome countings were made on temporary squash preparations.

The patients were treated with radium (50 mg. intra-uterine and 50 mg. in a vaginal box), and received, at the point from where the tissue for section was taken, a total dose of 21,000 r in three applications according to the modified Stockholm technique. The interval between the first and the second application was one week and between the second and third application was three weeks. Each time the radium was kept in position for 24 hours (total $2400 \times 3 = 7200$ mg. hours). About one month after completion of radium treatment, the dose received in the parametrium from radium application was supplemented by X-irradiation from a Million Volt X-ray unit (H.V.L. 3·3 mm. Pb 70 cm. F.S.D.). The total tumour dose delivered at each parametrium from the Million Volt unit was 3000 r.

One month after completion of radium irradiation, four or five pieces of tissue from different regions of cervix were again taken from each of the above selected patients for histological assessment of the radiotherapeutic effect. Tissues for histological study were fixed in Bouin's fluid and serial sections, which were stained in haematoxylin and eosin, were made through the whole tissue. These patients were kept under observation with repeated clinical examination every month (Tables II and III) up to a period of about thirty months from the date of the histological examination.

Cases in which the majority of the cells had an average chromosome number of more than sixty were taken as the higher ploidy group ; cases where the majority of the cells had an average chromosome number below sixty were taken as the lower ploidy group (Tables I, II and III).

TABLE I.—*Chromosomal Population of Malignant Cells of both Higher and Lower Ploidy Groups of Cases*

Case Number	Number of cells studied	Percentage of cells with chromosome numbers		Average chromosome number per cell containing	
		>60	<60	>60	<60
1	55	56·35	43·65	89·29 ± 33·92	49·00 ± 8·83
2	134	52·24	47·76	122·78 ± 54·79	41·66 ± 11·81
3	62	85·47	14·53	107·07 ± 21·50	37·22 ± 3·35
4	51	78·42	21·58	112·37 ± 31·70	33·63 ± 14·45
5	35	79·98	20·02	91·13 ± 36·40	38·14 ± 17·66
6	30	90·00	10·00	93·88 ± 5·38	48·00 ± 0
7	45	86·70	13·30	98·46 ± 33·92	43·66 ± 10·20
8	25	100·0	0	104·76 ± 50·14	0 ± 0
9	84	4·77	95·23	91·50 ± 21·56	28·87 ± 9·94
10	57	31·03	68·95	105·00 ± 60·89	42·43 ± 12·00
11	50	8·00	92·00	80·00 ± 16·00	38·73 ± 6·48
12	24	29·16	70·83	92·71 ± 43·06	49·82 ± 5·56
13	26	38·46	61·54	76·00 ± 12·61	38·23 ± 14·66
14	28	35·72	64·28	85·60 ± 16·85	46·44 ± 11·57
15	25	8·00	92·00	103·00 ± 00·00	41·75 ± 9·00
16	40	12·50	87·50	85·60 ± 6·16	45·20 ± 7·07
17	50	12·00	88·00	78·80 ± 15·59	41·00 ± 12·50
18	50	36·00	64·00	100·22 ± 41·01	46·06 ± 7·21
19	25	16·00	84·00	84·00 ± 18·76	45·09 ± 6·92
20	30	6·70	93·30	79·50 ± 23·32	49·03 ± 6·40

RESULTS

Out of twenty cases studied, in eight (Fig. 1) the majority of cells exhibited higher ploidy whereas the remaining twelve (Fig. 2) showed a lower ploidy. In the higher ploidy group, all patients exhibited persistence of malignant cells in their cervical biopsies examined histologically one month after completion of radium irradiation. Of these, six patients (75 per cent) died within a year after completion of radium treatment and two patients (25 per cent) are still (30 months after irradiation) living and in good condition so far as can be judged clinically (Table II). In the lower ploidy group, malignant cells were not found in the post-irradiation histological sections of all patients. Of these twelve patients, ten (83 per cent) are still living and in good condition while two patients (17 per cent) died about one year after completion of their treatment (Table III).

The age of the patients, type of local lesion with their clinical stages, degree of de-differentiation and the nature of stromal proliferation are differential neither with the chromosomal population nor with their radiobiological response.

TABLE II.—*Pre-radiation History and Post-radiation Clinical Check-up of Higher Ploidy Cases*

	Pre-radiation clinical notes				Post-radiation check-up		
Case	Age	Type of local lesion with stages	Broders's gradation and pearl formation	Stromal proliferation	Histological check-up (one month after radium therapy)	Clinical check-up up to 30 months after completion of radium therapy	Viability
1.	45	Proliferative III	II Pearl present	Scanty	Cancer cell present	—	Died after 8 months
2.	40	Ulcerative III	III Pearl present	,,	Ditto	Uterus normal up to 3rd month but local recurrence at 6th month	Died 12 months later.
3.	40	Proliferative IIA	III Pearl absent	Absent	,,	Anterior vaginal wall felt hard and a horse-shoe-like swelling on para-rectal tissue at 5th month	Died 18 months later.
4.	36	Proliferative III	Ditto	Scanty	,,	Local recurrence 1 month later	Died 8 months later.
5.	35	Proliferative II	II Pearl absent	,,	,,	Per-rectal and per-vaginal findings were normal 28 months later	Well 30 months later.
6.	50	Indurated growth III	III Pearl absent	Marked	,,	Local recurrence 5 months later	Died 7 months later.
7.	55	Ulcerative III	II Pearl absent	,,	,,	Both per-rectal and per-vaginal findings were normal 12 months later	Well 23 months later.
8.	40	Proliferative	Ditto	,,	,,	—	Died 6 months later.

TABLE III.—*Pre-radiation History and Post-radiation Clinical Check-up of Lower-ploidy Cases*

	Pre-radiation clinical notes				Post-radiation check-up		
Case	Age	Type of local lesion with stages	Broders's gradation and Pearl formation	Stromal proliferation	Histological check-up (one month after radium therapy)	Clinical check-up up tp 30 months after completion of radium therapy	Viability
9.	40	Ulcerative III	III Pearl absent	Scanty	Cancer cells not found	Per-vaginal finding was normal and there was a deep fibrosis in para-rectal tissue 18 months later	Well, 26 months later.
10.	29	Proliferative III	Ditto	,,	Ditto	Both per-vaginal and per-rectal findings were normal 25 months later	Well, 26 months later.
11.	30	Proliferative I	II Pearl absent	,,	,,	Both per-vaginal and per-rectal findings were normal 26 months later	Well, 26 months later.
12.	43	Indurated growth III	II Pearl present	Marked	,,	Both per-rectal and per-vaginal findings were normal 24 months later	Well, 24 months later.
13.	35	Proliferative II	II Pearl absent	,,	,,	Both per-rectal and per-vaginal findings were normal 19 months later	Well, 24 months later.
14.	67	Proliferative III	II Pearl present	Scanty	,,	Both per-rectal and per-vaginal findings were normal 12 months later	Well, 30 months later.
15.	60	Ditto	II Pearl absent	,,	,,	Per-vaginal finding was normal, vault was rough and both para-metria indurated up to 2nd month	Well, 27 months later.
16.	54	Indurated growth III	Ditto	Marked	,,	—	Died 11 months later.
17.	30	Ditto	III Pearl absent	Scanty	,,	Cervix was hard and regular and both para-rectal tissues were indurated and hard 6 months later	Died of dysentery 17 months later.
18.	48	Proliferative III	I Pearl present	Marked	,,	Both per-rectal and per-vaginal findings were normal 6 months later	Well, 19 months later.
19.	42	Indurated growth III	II Pearl absent	,,	,,	Both per-vaginal and per-rectal findings were normal 6 months later	Well, 19 months later.
20.	65	Ulcerative III	Ditto	,,	,,	Both per-vaginal and per-rectal findings were normal 6 months later	Well, 18 months later.

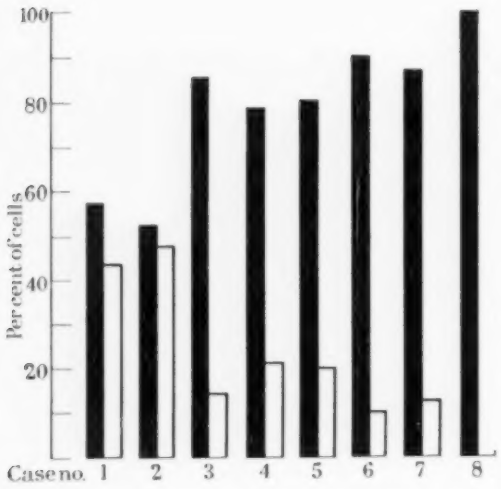

FIG. 1.—Histogram exhibiting chromosomal population of higher ploidy group of cases.
■ > 60 Chromosomes. □ < 60 Chromosomes.

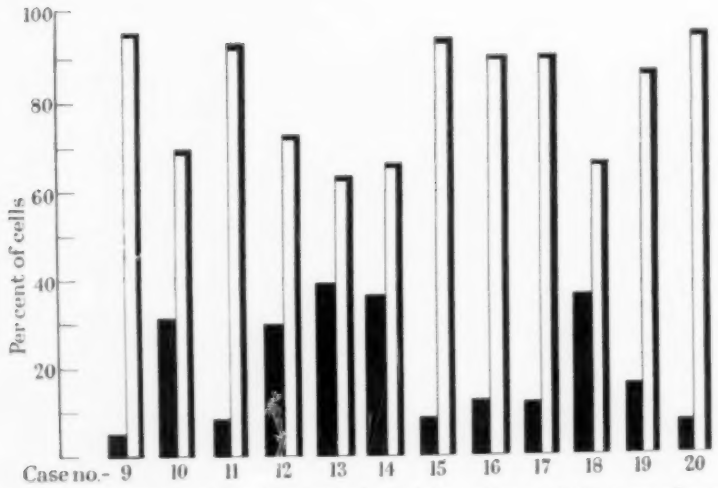

FIG. 2.—Histogram exhibiting chromosomal population of lower ploidy group of cases.
■ > 60 Chromosomes. □ < 60 Chromosomes.

DISCUSSION

In selecting proper biological material, solid tumour was considered to be more representative than the cells in tissue culture or in ascites cell tumour. In a tissue culture medium, the environmental condition of the cell is totally different from that of a cancer cell situated in the patient's body. Also, in ascites cell tumour, though *in vivo* condition is maintained yet, due to the absence of the stromal bed, the environmental condition is different. The ultimate therapeutic effect is due to the combined effect of the tumour cell and its stromal surroundings (Gricouroff, 1952). It is for this reason that, in the present investigation, solid tumour has been taken as a biological reference system.

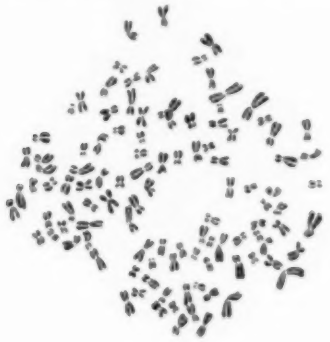

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FIG. 7 and 8.—Camera lucida drawings, exhibiting chromosomal populations of malignant cells in higher ploidy and lower ploidy groups of cases, respectively. (×1000.)

The difference between average chromosome number per cell of the higher ploidy and lower ploidy series was statistically analysed and the value of "t" was highly significant at 1 per cent level (Table IV).

TABLE IV.—*Evaluation of the Data by "t" Test of Higher and Lower Ploidy Groups of Cases*

Ploidy	Number of cases	Total number of cells	Mean chromosome number per cell with S.D.	Value of "t"	Significance
Higher ploidy groups of cases	8	437	$87 \cdot 64 \pm 25 \cdot 31$	21·64	Highly significant at 1 per cent level.
Lower ploidy groups of cases	12	489	$49 \cdot 77 \pm 27 \cdot 47$		

It has been found that there seems to be somewhat direct correlation between higher ploidy (75 per cent) of malignant cells and their radioresistant character. In two cases (cases 5 and 7) the correlation did not hold so far as thirty months survival of the patients was concerned (Table II).

In the lower ploidy group, the correlation with radiosensitivity was maintained in 83 per cent of the cases. In two cases (cases 16 and 17) the correlation was not observed (Table III).

SUMMARY

A correlation between radiosensitive and polyploidal behaviours of malignant cells *in vivo* was assessed in twenty cases of epidermoid carcinomata of the human cervix uteri.

It has been observed that in the majority of the cases (75 per cent), there is a correlation between higher ploidy (more than sixty chromosomes) of malignant cells and their radioresistant character.

There is also a direct relationship in the majority of cases (83 per cent) between lower ploidy (less than sixty chromosomes) of malignant cells with their radiosensitive behaviour.

I wish to express my deep gratitude to Professor S. Mitra, Director of the Chittaranjan National Cancer Research Centre, for his support and never failing interest in this investigation. I am also grateful to Dr. P. De, Head of the Department of Cell Research of this Institute and Dr. S. P. Raychaudhuri of the Animal Cytogenetics Laboratory of Calcutta University for their kind co-operation and guidance, failing which it would have been difficult for me to carry on this work. My thanks are due to Dr. S. Sur, Senior Registrar of Chittaranjan Cancer Hospital for following-up of cases. I am also indebted to my colleagues, Dr. T. Mukherjee and Mr. R. Chatterjee, who helped me in various ways in framing this paper.

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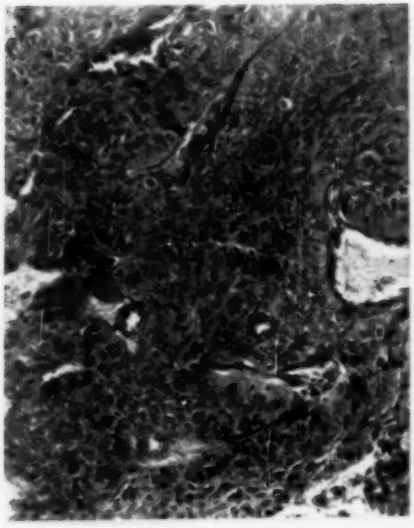
EXPLANATION OF PLATE

Fig. 3 and 4.—Sections of the tissues taken from the cervix of case 4 (higher ploidy) before and after irradiation of 21,000 r (respectively). (×140.)
Fig. 3.—Epidermoid carcinoma of cervix before irradiation.
Fig. 4.—Shows one of the serial sections taken from the cervix after irradiation, showing persistence of malignant cells in the tissues collected.
Fig. 5 and 6.—Sections of the tissues taken from the cervix of case 12 (lower ploidy) before and after irradiation of 21,000 r, respectively. (×140.)
Fig. 5.—Epidermoid carcinoma of cervix before irradiation.
Fig. 6.—Shows one of the serial sections taken from the cervix after irradiation, representing total absence of malignant cells from the tissues collected.



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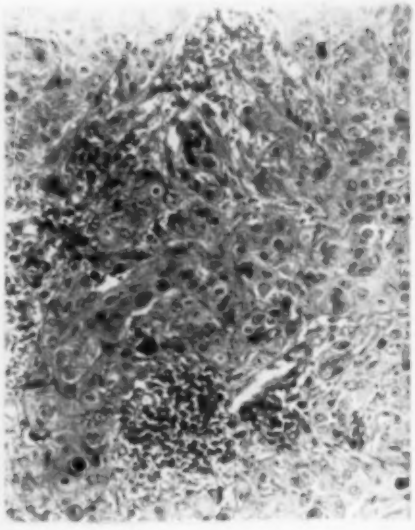

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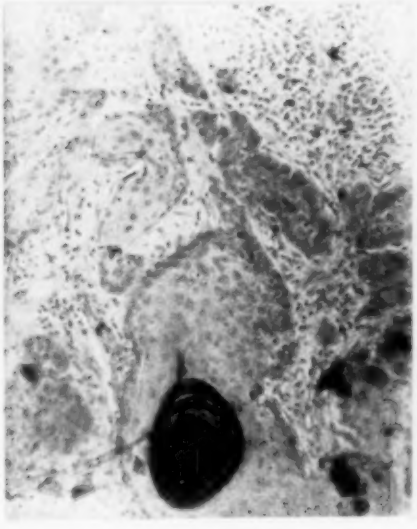

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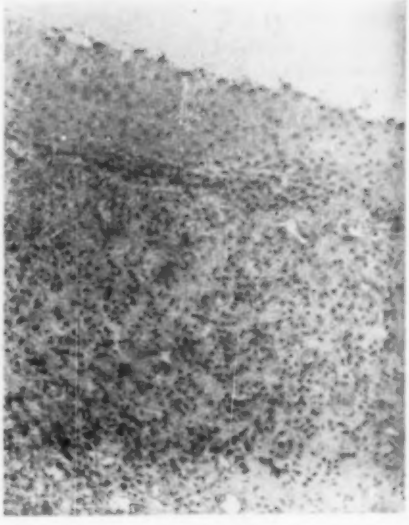

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EFFECT OF HIGH PRESSURE OXYGEN ON RADIOSENSITIVITY OF EHRLICH'S TUMOUR IN MICE AFTER "IMMUNOLOGICAL APPROXIMATION"

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THE radiosensitivity of both normal and neoplastic cells is increased if oxygen is present during irradiation (see Gray, 1958, for summary). On the assumption that tumours *in vivo* contain cells in a state of anoxia relative to normal tissues when air at atmospheric pressure is being respired by the host, breathing of pure oxygen at raised pressures during irradiation has been employed with a view to increasing the therapeutic ratio (Hollcroft, Lorenz and Mathews, 1952; Gray *et a.*, 1953; Dittrich and Stuhlman, 1954; Churchill-Davidson, Sanger and Thomlinson, 1957; du Sault, Eyler and Dobben, 1959). However, the validity of this hypothesis is difficult to establish experimentally and the problem has been discussed in recent reviews (Scott, 1958; Howard Flanders and Scott, 1960). Difficulties are encountered in immobilising conscious animals without causing compression of the tumour blood supply during irradiation. The need to employ anaesthetics and their side effects, adds further problems, as does the difficulty in providing adequate radiation dosimetry of such tumours. However, certain biological factors are of even greater significance. In using transplantable tumours, the homograft reaction complicates the situation by making radiocurability less difficult in certain circumstances. Cohen and Cohen (1960) have shown that even for an apparently isogenic situation concerning the C3H mouse mammary adenocarcinoma, previous immunological attenuation of the host by either whole body irradiation or corticosteroids, increases the LD_{50} curative X-ray dose from 5660 to 7500 rads and the estimated critical cell number (ED50) is reduced from 25 cells to approximately 1 cell. This suggests that in these experiments tumour and host were not, in fact, strictly isogenic, and some homograft reaction was operative.

Since the use of a readily available transplantable tumour, such as Ehrlich's ascites tumour has many technical advantages, experiments are reported in which this tumour was used as a homograft in hybrid mice after immunological approximation of the hosts, to determine the effect of high pressure oxygen respired by the treated animals, on tumour radiosensitivity and therapeutic ratio. This report includes initial experiments performed to determine the effect of whole body irradiation of inoculated mice on the "take" of Ehrlich's tumour.

MATERIALS AND METHODS

Animals and ascites tumour

Adult Walter and Eliza Hall hybrid mice weighing 40 g. were used for tumour inoculation. The mice were housed, six animals per cage, in an air conditioned

animal house at 21 ± 1° C. Different coloured paints were used as skin marks to distinguish groups of mice and individual mice, to allow cross caging, and scoring of tumour incidence, growth rates and tissue reactions.

The tumour was Ehrlich's ascites tumour (hyperdiploid line ELD Lettrè, 46 chromosome mode), henceforth called EAT, previously passaged at weekly intervals in C3H mice for over 3 years in these laboratories.

Inoculation

All inoculations were made in the hind limbs of mice, just proximal to the knee joint. For each inoculation a volume of 0·2 ml. of tumour cell suspension was slowly injected into the thigh muscle. To prepare the suspension, a seven day ascites growth of the tumour was harvested from donor mice. The cells for each sample were counted in a haemocytometer chamber, and a viability test performed using the eosin exclusion technique of Shrek according to the method of Hoskins, Meynell and Sanders (1956); samples in which more than 5 per cent of cells stained pink were discarded. Appropriate dilutions of EAT cells for inoculation were made in ice cold Tyrode solution, to give cell concentrations ranging from 10^1–10^6 EAT cells per 0·2 ml. inoculum.

Preparation of recipient mice prior to inoculation

One of three methods of preparing recipient mice were used :—

(*a*) No treatment (immunologically competent group)

(*b*) Chronic depletion of endogenous amines with Compound 48/80, given for 9 days prior to EAT inoculation. Increasing doses were administered twice daily in accordance with footnote to Table I.

(*c*) Whole body X-irradiation in doses of 400 r or 500 r given 24 hours preceding inoculation with EAT cells. The radiation was delivered by a constant potential X-ray source operated at 250 kv, 15 mA, 30 cm. FSD, and 1 mm. Cu HVL giving a dose rate in air of 300 r per minute.

Irradiation of tumour cells in vivo.

Groups of untreated and whole body irradiated recipient mice were inoculated with donor cells, previously exposed to graded doses of irradiation *in vivo*. The latter was accomplished by taking 10 donor mice with a ten day ascitic growth of EAT, and subjecting the animals to whole body irradiation. Viability counts (eosin exclusion index) were performed to check donor fluids before irradiation, and the unirradiated samples were pooled and titrated in groups of mice. The irradiation factors used to deliver whole body irradiation to these ascitic donor mice were 250 kv, 15 mA, 30 cm. FSD, 1 mm. Cu HVL giving a dose rate in air of 300 r per minute. Before irradiation the ten mice were placed in the same container which was surrounded with bolus to give full back scatter. The radiation exposure was interrupted after 200, 400, 800, 1600 and 2400 r had been administered, so that 2 mice could be removed and samples of tumour fluid withdrawn, counted, pooled and diluted for titration in groups of recipient mice. At each interruption of the exposure, the two mice selected at random were removed, marked, ascitic fluid withdrawn, the animals replaced and the exposure continued. A pair of different animals was used for each successive removal of irradiated cells.

TABLE I.—*Effect of Sub-lethal Whole Body X-irradiation (400 r) and Histamine Depletion (Compound 48/80 for 8 Days)* on Growth Rate and "Take" of EAT Cells in Female Hybrid Mice*

Inoculum size EAT cells	Group	Mean increase in circumference of leg (cm.) (Days after inoculation) 7	14	21	28	Fraction recipients with tumours 8 weeks after inoculation†
10^6	Unirradiated	1·4	2·4	3·3	4·4	6/6
	Irradiated	1·1	2·4	3·5	4·8	6/6
	48/80	1·5	2·7	3·3	4·3	6/6
10^5	Unirradiated	0·8	2·0	3·0	4·4	6/6
	Irradiated	0·8	2·2	3·2	4·3	6/6
	48/80	0·7	2·2	3·3	3·8	6/6
10^4	Unirradiated	0·3	0·7	0·8	1·3	5/6
	Irradiated	0·3	0·9	1·5	2·2	6/6
	48/80	0·4	0·6	1·2	1·6	4/5
10^3	Unirradiated	0·2	0·3	0·4	0·9	4/6
	Irradiated	0·2	0·5	1·0	1·2	5/6
	48/80	0·1	0·3	0·4	0·6	2/6
10^2	Unirradiated	0·2	0·3	0·1	0·3	1/6
	Irradiated	0·2	0·3	0·7	1·1	5/6
	48/80	0·1	0·3	0·2	0·3	1/6

* Dose schedule compound 48/80 (intraperitoneal injections) was as follows: Day (1) 1 × 30 μg.; (2) 2 × 40 μg.; (3) 2 × 50 μg.; (4) 2 × 60 μg.; (5) 2 × 70 μg.; (6), (7) and (8) 1 × 80 μg.; and inoculations on day 9.

† Mice developing large tumours during the observation span were sacrificed after 4 weeks but scored as positive.

Local irradiation of tumours—oxygen pressurisation

In these experiments each inoculation consisted of 10^6 EAT cells injected into the right thigh of the mouse. The tumour was allowed to grow for 7–8 days, by which time the limb circumference had increased by approximately 10 mm. and contained a hard palpable tumour which distended the thigh as a spindle-shaped swelling. Calculations based on this average increase in size of limbs gives a tumour volume of approximately 1 c.c. at the time of irradiation. In over 1000 consecutive mice inoculated with 10^5 or more EAT cells a palpable tumour was present in each animal 7–8 days after inoculation and no spontaneous regressions have been observed to date, whether recipient mice were untreated or "immunologically attenuated" as the result of whole body irradiation. A few mice in which tumours were considered undersize or oversize at 7–8 days, were rejected, as were mice in which the tumour had extended beyond the thigh into the buttock, so that its inclusion in the radiation field could not be ensured.

Mice were anaesthetised with sodium pentobarbital (70 mg. per kg. injected intraperitoneally) and irradiated in a steel pressure vessel of 35 litres capacity. The X-ray beam passed through a circular perspex portal 2·5 cm. thick, occupying the central portion of the upper lid of the chamber, which was retained in position by clamps when the chamber was pressurised. After decompression the clamps could be loosened and the lid elevated on guides and locked in position to introduce or remove animals from the chamber. To the undersurface of the lid an elevation

platform was attached on which animal containers could be placed and raised to proximity with the undersurface of the perspex portal. The mouse tumours were irradiated in a special perspex container (see cross-section shown in Fig. 1). The animal body was placed in the outer well (a), and shielded with lead. This well contained 4 animals at each irradiation. The tumour bearing limb protruded through a semi-circular aperture in the lead shielding, into the central well, and was gently extended and fixed by a silk thread suture to a central pillar. The thread suture transfixed a 3 mm. wide strip of elastoplast, wrapped around the foot with minimal compression. The central well containing the 4 limbs was filled with bolus and the whole container placed beneath the perspex portal of the pressure vessel as shown. The top of the chamber was lowered, clamped in the closed position and the tumours irradiated through the perspex portal, whilst the chamber contained either air at atmospheric pressure or pure oxygen under pressure. The radiation apparatus used generated X-rays at 250 kv, 15 mA, 30 cm. FSD, 1 mm. Cu HVL giving a dose rate in air of 300 r per minute. The mean tumour dose (TD) was calculated at point y (Fig. 1) and calculations checked by direct measurements with ionisation chambers placed at points a, x, y and z as shown. The calculated and measured values were in close agreement. The tumour doses used varied from 500 r to 4000 r and were given as single exposures. The variation in dosage with depth in the tumour was less than ± 6 per cent. However, animals received a whole body dose contribution (WBD), measured at point a, approximately 8·5 per cent of the mean TD, giving the following relationship between TD and WBD :—

TD (*r*)	500	1000	2000	3000	4000
WBD (*r*)	42	85	170	255	340

This whole body contribution resulting from irradiation of tumours must be added to the whole body dose (400 r) received 8 days previous (24 hours before inoculation).

During irradiation of tumours in animals breathing air at atmospheric pressure, a slow flow of air through the chamber was maintained. For irradiation of animals in oxygen under pressure, the chamber was flushed out with pure oxygen until the percentage oxygen concentration, measured on samples of exhaust gases with a Beckman Model D2 oxygen analyser, exceeded 95 per cent. Compression followed at a rate of 15 lb. per square inch per minute, till a preset level of 45 lb. per square inch gauge pressure (4 atmospheres absolute) was reached. This pressure was maintained in the apparatus during irradiation. Decompression, also at 15 lb. per square inch per minute, followed immediately after completing the radiation exposure. The ambient temperature in chamber changed by less than 1·5° C. during compression and decompression. Provision was not made for absorption of CO_2 within the chamber and was considered unnecessary owing to the negligible amount which accumulated. The chamber was provided with a series of electrically insulated contacts to allow polarographic measurements of oxygen tension to be made in tissues of animal under pressure (*vide infra*).

Polarographic measurements of oxygen tension in tumours

Since the tension of molecular oxygen which exists in the tumour is of paramount importance to the rationale of respiring oxygen at increased pressures as

an adjunct to radiotherapy of tumours, polarographic measurements were made of oxygen tension in tumours and normal tissues of animals respiring oxygen at different pressures. Open type cathodes were made from platinum wire, 32 s.w.g., 0·23 mm. in thickness, and covered with Araldite insulation except for the terminal 1 mm. tip. These were inserted to various depths in the tumour, or in surrounding normal tissues. The circuit included a galvanometer, of variable sensitivity, used to measure the cathode current. The latter is considered to represent the oxygen tension adjacent to the exposed platinum surface (Davies and Brink, 1942). The circuit was completed using an anode consisting of Pb/standard acetate solution/KCl-Agar bridge (Tödt *et al.*, 1952), inserted in the rectum or subcutaneous tissues of the animal. Previous experiments to calibrate this system showed a plateau in the current-voltage calibration curve similar to that for the calomel half-cell anode (Davies and Brink, 1942). For the latter the mid-region of the plateau corresponded to an impressed potential of −0·60 volt, but for the Tödt anode this region corresponds to zero potential. The electrodes were calibrated in Hank's solution containing 20 per cent v/v sheep serum, equilibrated with various tensions of oxygen. The form of the oxygen-current curves obtained agreed with those of Harris and Barclay (1955) for O_2 tensions below 760 mm. Hg, with a linear relationship of the logarithm of the O_2 tension (T) to the current (I), such that

$$\log T = K_1 I + K_2$$

where K_1, K_2 are constants determined by the characteristics of the Pt electrode used. For O_2 tensions in excess of 760 mm. Hg, determined inside the pressure chamber at an ambient temperature of 19°C the relationship between T and I was essentially linear. However, conditions for calibration under pressure, could not be readily controlled in respect to temperature and water vapour pressure. Furthermore the time allowed (10 minutes) for equilibration of the calibration fluid with the gas phase may have been too short although the current had reached a maximum after 5 minutes using 2 ml. of fluid to give a depth of 3 mm. in a dish 3 cm. in diameter. The pressure vessel was rocked to give quite vigorous agitation of the fluid between measurements, taken at 2 minute intervals. Currents obtained for electrodes in tissues, showed great variability, according to the site and positioning of electrodes and their movements in tissue. Calibrated electrodes gave tissue oxygen tensions considerably in excess of values determined by other means for tissue fluids. However, the response of electrodes to changes in respired oxygen tension was consistent, being unaffected by moderate elevations in respired CO_2 and certain treatments not considered to interfere with tissue oxygen tensions. The relative changes in the current registered by electrodes placed in the tumour and normal tissues of anaesthetised mice respiring different tensions of O_2 were considered of sufficient significance to warrant the inclusion of representative records under results, together with additional details of the experimental technique.

Tests for vascular compression

Since any reduction in blood flow in the limb during irradiation resulting from positioning of the animal, would reduce the effects of an increase in respired oxygen tension, special care was taken to avoid stretching and compression of the limbs during treatment. At no stage was pallor or cyanosis noted in the skin of extended limbs. With the legs of anaesthetised mice held in the irradiation position (Fig. 1),

an electrode was inserted in the popliteal region of each extended leg. The oxygen current was registered with animals successively breathing air, pure O_2, 5 per cent O_2 and air and faithful changes in current resulted. A further test was performed with anaesthetised mice in the irradiation position. Two ml. of lissamine green (Gurr No. 3591) solution was injected intraperitoneally, and the time of appearance of the green discoloration of the skin of extended and unextended limbs measured. No significant difference was observed for the two limbs nor were the results in unanaesthetised mice significantly different from those in anaesthetised animals.

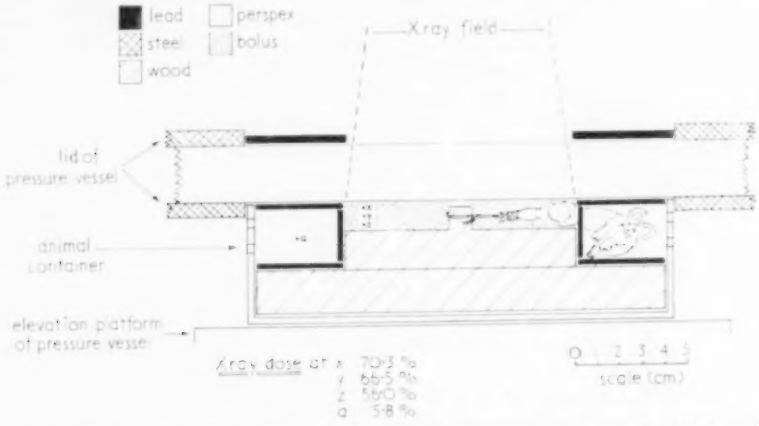


FIG. 1.—Cross section through irradiation portal of pressure chamber and container for irradiating mouse legs (tumours). Percentage depth doses measured at points *a*, *x*, *y* and *z* with shielding and bolus in position are shown. Outer well of container normally holds four mice at a time during irradiation exposure, but only one is shown.

Tumour regression

Results of treatment were assessed either as *cure* (no palpable tumour) 8 weeks after irradiation or as *growth rate* (circumference of limb) during 4 weeks after irradiation. Limbs of survivors were palpated weekly after irradiation, and presence or absence of palpable tumour charted. Mice dying within 14 days of irradiation of tumours, were excluded from the analysis, after which time a progressive record of the fraction of animals with tumours was made weekly. Mice surviving at 6 weeks with tumours 40 mm. or more in circumference were killed and scored as failed cures at 8 weeks. Mice in which the irradiated tumour disappeared, but extension appeared proximal to the irradiated zone were scored as failed cures.

The maximum limb circumference at the site of the tumour was measured by means of a silk loop protruding from the end of a transfusion needle and held taut by a loose trocar fitted inside the canula. The loop was withdrawn from the limb and the doubled length of silk measured against a rule. This method has proved much more reproducible than the use of calipers in measuring size of limb tumours.

Tissue reactions

A time 4 weeks after irradiation was chosen to assess tissue reactions in irradiated limbs, which were scored and given an arbitrary numerical index of severity

as follows :—

Partial epilation	1
Complete epilation	2
Superficial skin loss	3
Skin loss and oedema	4
Partial sloughing (below knee)	5
Partial sloughing (below and above knee)	6
Complete sloughing (" radiation amputation ")	7

Tissue reactions in mice irradiated in oxygen under pressure (OHP) and air corresponding to each dose, were compared by calculating the mean " severity index ", X_{OHP} and X_{air} respectively, and using the ratio,

$$t = \frac{X_{OHP}}{X_{air}}$$

where t is the " oxygen effect factor " for each dose level.

Statistical analysis

Probit analysis (Finney, 1952) was used to calculate regression equations, and to determine the significance of observed differences in cure rate in animals breathing air, and oxygen under pressure. Other statistical procedures such as the calculation of variances, students t values and the χ^2 test, were carried out in accordance with standard practice.

RESULTS

Inoculation of irradiated and unirradiated hosts

(a) *Unirradiated cells.*—Limbs of mice were inoculated with graded numbers of EAT cells in $\log_{10}$ dilutions from 10^6 to 10 cells, and the tumours scored which developed during a period of 50 days after inoculation. The number of cells required to produce 50 per cent of tumours ($ED_{50/50}$ days) was calculated from probit regression curves. In untreated mice the $ED_{50/50}$ was 281 ($\pm$71 SE) cells ; in mice irradiated with 500 r whole body X-ray dose 24 hours before inoculation the $ED_{50/50}$ was reduced to <10 cells. Increase of the irradiation-inoculation interval to 7 days increased the $ED_{50/50}$ to 31 ($\pm$18) cells.

To determine the effect of " immunological attenuation " by whole body irradiation on the growth rate of EAT in legs of mice, tumour cells were titrated in the limbs of (i) unirradiated mice ; (ii) mice receiving 400 r, 24 hours preceding inoculation, and (iii) mice treated with increasing doses of compound 48/80 for 8 days preceding inoculation, to deplete tissue histamine (Feldberg and Talesnik, 1953). This last group was included for two reasons ; firstly to act as a further control group, and secondly in view of a previous finding that in chronic histamine depleted rats a heterologous graft of Ehrlich's tumour grew more rapidly, as a temporary graft after inoculation (van den Brenk and Upfill, 1958). The limb sizes in the various groups were measured weekly for 4 weeks after inoculation. Also the total incidence of tumours which had developed in 8 weeks was recorded. The results are given in Table I, Fig. 2 and show that for larger inocula (10^5, 10^6 cells) the mean rate of tumour growth (and tumour incidence) is affected neither

by whole body irradiation nor by histamine depletion. However, for smaller inocula ($<10^4$ cells), the mean rate of growth was clearly increased by whole body irradiation, but not by histamine depletion, and was paralleled by an increase in tumour incidence. However, the growth curves show how difficult it is to relate quantitatively, growth rate of a tumour *in vivo*, to the number of viable cells initiating such growth. Furthermore, the standard deviations for measurements of tumours resulting from small inocula, were much greater than for larger inocula, owing to tumours not developing in some animals, whilst in remaining animals tumours often grew almost as rapidly as those in animals which had received much larger inocula.

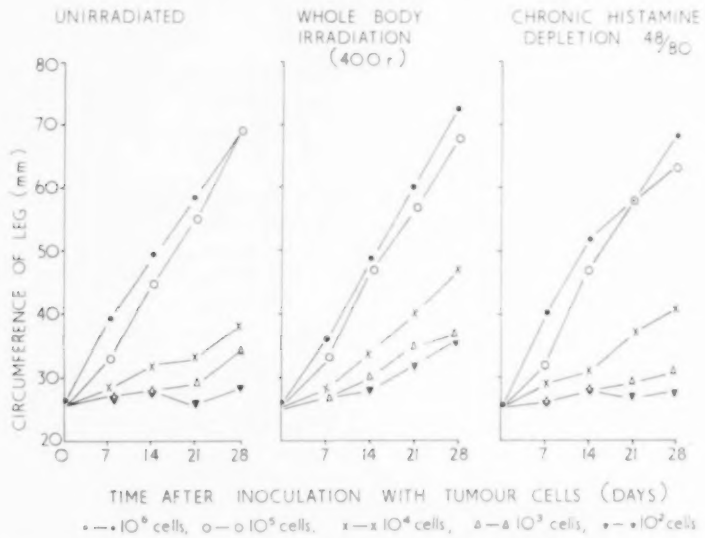


FIG. 2.—Growth curves for Ehrlich's tumour in mouse limbs, following titration of cells in untreated mice, mice previously treated with Compound 48/80 ("subacute depletion", see text) and mice which received 400 r whole body X-irradiation 24 hours before inoculation.

(b) *Irradiated cells.*—Parallel numbers of EAT cells were irradiated with doses of X-rays *in vivo*, and inoculated into the limbs of either unirradiated mice or mice which had received 500 r whole body irradiation, 24 hours preceding inoculation. The results are set out in Table II, and show a marked increase in survival of EAT cells in irradiated recipient mice. Calculation of the doses (LD_{50}) received by EAT cells *in vivo* to give 50 per cent incidence of tumours in mice for respective sizes of inoculum are set out in Table III. The LD_{50} dose was approximately 3·5 times higher if irradiated (immunologically attenuated) recipient mice were used. In other words, the radiation dose necessary to sterilise EAT cells *in vivo* may be underestimated by a factor of 3·5 in the homologous situation used in this experiment, if the recipient mice are untreated, in comparison to "immunologically attenuated" mice.

TABLE II.—*Titration of Ehrlich Ascites Tumour Cells Irradiated* In Vivo *in ascitic Form, in Mice Subjected to* (a) *no Previous Irradiation, and* (b) 500 *r Whole Body Irradiation* 24 *hours Preceding Inoculation. The Cells were Inoculated into Legs of Recipient Mice, and Tumour Incidence Scored at* 53 *Days**

Number of cells inoculated	Irradiation dose *in vivo*	Tumour incidence—fraction (%)	
		Untreated recipients	Irradiated recipients
10^2	0 r	5/12 (42)	9/9 (100)
	200 r	4/8 (50)	8/8 (100)
	400 r	1/8 (12)	4/6 (67)
	800 r	2/8 (25)	3/5 (60)
	1600 r	0/8 (0)	1/8 (12)
	2400 r	0/8 (0)	0/8 (0)
10^3	0 r	9/10 (90)	8/8 (100)
	200 r	3/5 (60)	8/8 (100)
	400 r	4/8 (50)	7/7 (100)
	800 r	3/8 (37)	4/7 (57)
	1600 r	1/8 (12)	5/8 (62)
	2400 r	0/8 (0)	1/8 (12)
10^4	0 r	20/20 (100)	12/12 (100)
	200 r	6/7 (86)	8/8 (100)
	400 r	8/8 (100)	7/7 (100)
	800 r	8/8 (100)	3/3 (100)
	1600 r	2/8 (25)	7/7 (100)
	2400 r	0/8 (0)	6/8 (75)
10^5	0 r	28/28 (100)	10/10 (100)
	200 r	8/8 (100)	7/7 (100)
	400 r	8/8 (100)	8/8 (100)
	800 r	8/8 (100)	6/6 (100)
	1600 r	6/8 (75)	8/8 (100)
	2400 r	7/8 (87)	7/7 (100)

* Recipient mortality over 53 days after inoculation 8/234 = 2·6 per cent for unirradiated mice, 35/216 = 16 per cent for irradiated mice.

TABLE III.—LD_{50} *Doses for* 10^2–10^5 *Cell Inocula in Non-Irradiated and Irradiated* (500 *r*) *Recipient Mice Respectively* (*from Data Table II*). *Dosage Reduction Factor* R *calculated from*

$$R = \frac{LD_{50}}{LD_{50}}\left(\frac{\text{irradiated recipients}}{\text{non-irradiated recipients}}\right)$$

Inoculum size of EAT cells	Recipients	LD_{50}	*R*
10^2	Non-irradiated	200 r	3·6
	Irradiated	720 r	3·6
10^3	Non-irradiated	340 r	3·5
	Irradiated	1200 r	3·5
10^4	Non-irradiated	1050 r	—
	Irradiated	(insufficient data)	—
10^5	Non-irradiated	(insufficient data)	—
	Irradiated	(insufficient data)	—

by whole body irradiation nor by histamine depletion. However, for smaller inocula ($<10^4$ cells), the mean rate of growth was clearly increased by whole body irradiation, but not by histamine depletion, and was paralleled by an increase in tumour incidence. However, the growth curves show how difficult it is to relate quantitatively, growth rate of a tumour *in vivo*, to the number of viable cells initiating such growth. Furthermore, the standard deviations for measurements of tumours resulting from small inocula, were much greater than for larger inocula, owing to tumours not developing in some animals, whilst in remaining animals tumours often grew almost as rapidly as those in animals which had received much larger inocula.

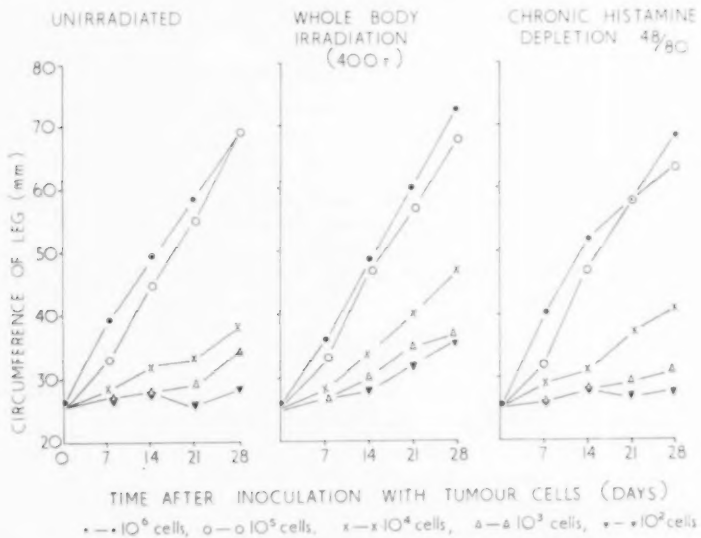


FIG. 2.—Growth curves for Ehrlich's tumour in mouse limbs, following titration of cells in untreated mice, mice previously treated with Compound 48/80 ("subacute depletion", see text) and mice which received 400 r whole body X-irradiation 24 hours before inoculation.

(b) *Irradiated cells.*—Parallel numbers of EAT cells were irradiated with doses of X-rays *in vivo*, and inoculated into the limbs of either unirradiated mice or mice which had received 500 r whole body irradiation, 24 hours preceding inoculation. The results are set out in Table II, and show a marked increase in survival of EAT cells in irradiated recipient mice. Calculation of the doses (LD_{50}) received by EAT cells *in vivo* to give 50 per cent incidence of tumours in mice for respective sizes of inoculum are set out in Table III. The LD_{50} dose was approximately 3·5 times higher if irradiated (immunologically attenuated) recipient mice were used. In other words, the radiation dose necessary to sterilise EAT cells *in vivo* may be underestimated by a factor of 3·5 in the homologous situation used in this experiment, if the recipient mice are untreated, in comparison to "immunologically attenuated" mice.

TABLE II.—*Titration of Ehrlich Ascites Tumour Cells Irradiated* In Vivo *in ascitic Form, in Mice Subjected to* (a) *no Previous Irradiation, and* (b) *500 r Whole Body Irradiation 24 hours Preceding Inoculation. The Cells were Inoculated into Legs of Recipient Mice, and Tumour Incidence Scored at 53 Days**

Number of cells inoculated	Irradiation dose *in vivo*	Tumour incidence—fraction (%)	
		Untreated recipients	Irradiated recipients
10^2	0 r	5/12 (42)	9/9 (100)
	200 r	4/8 (50)	8/8 (100)
	400 r	1/8 (12)	4/6 (67)
	800 r	2/8 (25)	3/5 (60)
	1600 r	0/8 (0)	1/8 (12)
	2400 r	0/8 (0)	0/8 (0)
10^3	0 r	9/10 (90)	8/8 (100)
	200 r	3/5 (60)	8/8 (100)
	400 r	4/8 (50)	7/7 (100)
	800 r	3/8 (37)	4/7 (57)
	1600 r	1/8 (12)	5/8 (62)
	2400 r	0/8 (0)	1/8 (12)
10^4	0 r	20/20 (100)	12/12 (100)
	200 r	6/7 (86)	8/8 (100)
	400 r	8/8 (100)	7/7 (100)
	800 r	8/8 (100)	3/3 (100)
	1600 r	2/8 (25)	7/7 (100)
	2400 r	0/8 (0)	6/8 (75)
10^5	0 r	28/28 (100)	10/10 (100)
	200 r	8/8 (100)	7/7 (100)
	400 r	8/8 (100)	8/8 (100)
	800 r	8/8 (100)	6/6 (100)
	1600 r	6/8 (75)	8/8 (100)
	2400 r	7/8 (87)	7/7 (100)

* Recipient mortality over 53 days after inoculation 8/234 = 2·6 per cent for unirradiated mice, 35/216 = 16 per cent for irradiated mice.

TABLE III.—*LD_{50} Doses for 10^2–10^5 Cell Inocula in Non-Irradiated and Irradiated (500 r) Recipient Mice Respectively (from Data Table II). Dosage Reduction Factor* R *calculated from*

$$R = \frac{LD_{50}}{LD_{50}} \left(\frac{\text{irradiated recipients}}{\text{non-irradiated recipients}} \right)$$

Inoculum size of EAT cells	Recipients	LD_{50}	*R*
10^2	Non-irradiated	200 r	3·6
	Irradiated	720 r	3·6
10^3	Non-irradiated	340 r	3·5
	Irradiated	1200 r	3·5
10^4	Non-irradiated	1050 r	—
	Irradiated	(insufficient data)	—
10^5	Non-irradiated	(insufficient data)	—
	Irradiated	(insufficient data)	—

Effect of high pressure oxygen (OHP) on radiosensitivity

(a) *Curative results.*—Previous experiments using untreated host mice, gave very variable dose-effect curves for the cure of solid Ehrlich's tumour by X-radiation, and in the present experiment, all mice received 400 r whole body X-radiation 24 hours preceding inoculation of limbs with 10^6 EAT cells. The apparent cure rate of tumours at various intervals after local irradiation of 7–8 day old tumours in anaesthetized mice breathing either air at atmospheric pressure or pure oxygen at 45 lb. per square inch gauge pressure (4 atmospheres absolute) is given in Table IV. The overall cure rate (52 per cent) estimated at 8 weeks after irradiation for mice breathing OHP was significantly better ($p < 0{\cdot}001$) than that (18 per cent) for animals breathing air.

TABLE IV.—*Resolution of Palpable Tumours (Cure Rate) in Hind Limbs of Inoculated Mice Following X-irradiation. All Animals Received 400 r Whole Body Irradiation 24 Hours Before Inoculation with 10^6 Ehrlich Ascites Tumour Cells, and the Growing Tumours were Irradiated under Pentobarbital Anaesthesia 7 or 8 Days Later with Mice Breathing Either Air at Atmospheric Pressure, or Pure Oxygen at 45 lb. per Square Inch Gauge Pressure (OHP)*

Tumour dose and gas respired	Fraction cured (%) at stated times after irradiation				Fraction of mice (%) dead from 2 to 6 weeks after irradiation
	14 days	28 days	6 weeks	8 weeks	
500 r in air	0/5 (0)	0/5 (0)	0/3 (0)	0/3 (0)	2/5 (40)
500 r in OHP	0/10 (0)	0/9 (0)	0/8 (0)	0/8 (0)	2/10 (20)
1000 r in air	0/39 (0)	1/20 (5)	1/17 (6)	1/15 (7)	22/39 (56)
1000 r in OHP	3/42 (7)	3/19 (16)	3/11 (27)	3/8 (37)	31/42 (74)
1500 r in air	1/27 (6)	1/19 (5)	1/15 (7)	1/15 (7)	12/27 (44)
1500 r in OHP	13/28 (46)	8/19 (42)	6/12 (50)	4/10 (40)	16/28 (57)
2000 r in air	4/37 (11)	3/33 (9)	4/28 (14)	3/24 (12)	9/37 (24)
2000 r in OHP	28/37 (76)	19/30 (63)	13/23 (56)	12/22 (55)	14/37 (38)
3000 r in air	12/43 (51)	16/35 (46)	11/27 (41)	6/24 (25)	16/43 (37)
3000 r in OHP	16/26 (62)	16/19 (84)	10/12 (83)	8/10 (80)	14/26 (54)
4000 r in air	6/10 (60)	8/9 (89)	8/9 (89)	5/6 (83)	1/10 (10)
4000 r in OHP	6/10 (60)	10/10 (100)	7/7 (100)	6/6 (100)	3/10 (30)
All doses in air	33/161 (20)	29/121 (24)	25/99 (25)	16/87 (18)*	62/161 (39)†
All doses in OHP	66/153 (43)	56/106 (53)	39/73 (55)	33/64 (52)*	80/153 (52)†

* Total lethalities, 8 weeks after irradiation were 41·8 per cent (71/170) for mice treated in air, and 55·7 per cent (92/165) for mice treated in OHP. The difference is probably significant ($\chi^2 = 3{\cdot}5$, $p = 0{\cdot}05$) as is that for total deaths occurring between second and sixth weeks† ($\chi^2 = 3{\cdot}4$, $p = 0{\cdot}05$).

† Total cure rate significantly higher in OHP ($\chi^2 = 10{\cdot}9$, $p < 0{\cdot}001$).

Calculation of probit regression equations for the log dose-effect relationship for the two groups, gave regressions which were essentially parallel and given by—

$$y = 3{\cdot}40x + 1{\cdot}94 \quad \text{(air)}$$
$$y = 3{\cdot}40x + 3{\cdot}22 \quad \text{(OHP)}$$

Comparison of the regressions, gives a dosage reduction factor of 2·4 (range of one

standard error is 2·9, 1·9). The calculated regressions are plotted on a linear scale in Fig. 3.

In this experiment, 335 tumours were irradiated, 170 in air and 165 in OHP respectively. Six weeks after irradiation of tumours, only 99 animals survived in the groups treated in air, and 73 in OHP treated groups, giving lethalities of 41·8 and 55·7 per cent respectively 6 weeks after irradiation of tumours. The high lethality rate is variously attributed to whole body irradiation, severe local tissue

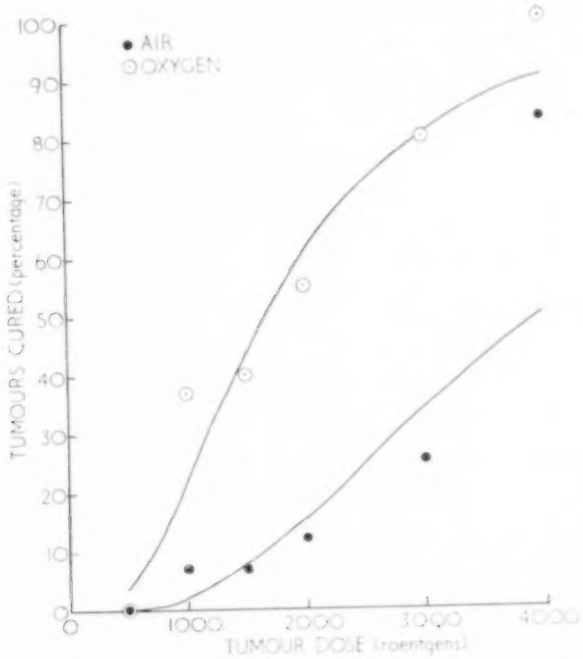


FIG. 3.—Calculated regression curves for percentage cure rate at 8 weeks after irradiation of Ehrlich's tumour in " immunologically attenuated " mice breathing either air (atmospheric pressure) or pure oxygen (at 45 lb. per square inch gauge pressure) during irradiation.

reactions in higher dosage groups, the toxic effects of spontaneous tumour necrosis and to a lesser extent metastases which were observed in approximately 2 per cent of the treated mice. Whilst the lethality was higher in the OHP treated group, despite the higher cure rate of tumours in surviving animals, this increase is not considered largely due to any toxic effects of oxygen (e.g. pulmonary damage). The high radiation dose rate used enabled exposure of animals to OHP to be kept to a minimum and barbiturate anaesthesia also provides marked protection against oxygen poisoning (Bean, 1945). In our animals convulsions under OHP did not occur and very few deaths were recorded within 72 hours of exposure to OHP. Local tissue reactions were more severe in this group (*vide infra*) and are considered a more important contribution to lethality than oxygen poisoning.

However, careful post mortem examinations to ascertain causes of death were not performed.

(b) *Effects on tumour growth rate.*—The size of tumours after irradiation, were noticeably smaller in the OHP treated groups. In Table V measurements made

TABLE V.—*Measurements of Circumferences of Normal (Left) Limbs and Right Limbs Inoculated with* 10^6 *Cells of Ehrlich Ascites Tumour, Before and After X-irradiation of Tumours. Mice Breathing Either Air or Pressurised Oxygen, OHP (45 lb. per Square Inch Gauge Pressure) During Irradiation. All Animals Received 400 r Whole Body Irradiation 24 Hours Preceding Inoculation with Tumour Cells. Tumours Locally Irradiated 7 Days After Inoculation*

Irradiation treatment (number of mice)	Side	Limb circumference ± SE in mm. at times (days) after inoculation				Surviving fraction mice with palpable tumours (28 days after inoculation)
		7	14	21	28	
Unirradiated	R.	34 ± 0·6	44 ± 1·0	52 ± 3·4	68 ± 4·7	14/14
(15)	L.	24 ± 0·3	..	..	..	
500 r in air	R.	35 ± 0·6	46 ± 0·9	..	>60	8/8
(9)	L.	24 ± 0·6	..	..	..	
500 r in OHP	R.	33 ± 0·7	43 ± 1·0	..	>60	8/8
(8)	L.	25 ± 0·4	..	..	..	
1000 r in air	R.	32 ± 0·6	39 ± 1·7	39 ± 1·5	42 ± 2·3	9/9
(10)	L.	23 ± 0·4	..	..	..	
1000 r in OHP	R.	33 ± 0·8	30 ± 0·9	31 ± 0·7	35 ± 3·4	5/7
(9)	L.	24 ± 0·4	..	..	..	
2000 r in air	R.	36 ± 1·0	33 ± 1·2	38 ± 1·6	42 ± 3·4	7/10
(10)	L.	26 ± 0·5	..	..	..	
2000 r in OHP	R.	35 ± 0·9	31 ± 0·9	31 ± 1·0	32 ± 1·0*	3/9
(10)	L.	25 ± 0·5	..	..	..	
3000 r in air	R.	34 ± 0·6	32 ± 1·5	..	..	3/9
(10)	L.	24 ± 0·4	..	..	..	
3000 r in OHP	R.	33 ± 0·8	30 ± 1·0	..	..	0/8
(10)	L.	25 ± 0·4	..	..	..	
4000 r in air	R.	32 ± 0·6	30 ± 0·9	..	..	1/9
(10)	L.	24 ± 0·3	..	..	..	
4000 r in OHP	R.	34 ± 0·5	31 ± 0·6	..	..	0/7
(10)	L.	24 ± 0·5	..	..	..	

* Severe skin reactions in 3/9 mice, prevented measurement of limbs. In 3000 r and 4000 r groups severity of reactions also prevented measurements of 21 and 28 day tumour sizes.

weekly for 3 weeks after irradiation are recorded for tumour doses ranging from 500 to 4000 r, administered to mice in either air or OHP. It is seen that repeated measurements over this 3 week period, could only be made with any accuracy for doses less than 2000 r owing to local tissue reactions. In Fig. 4 growth curves are plotted for control (unirradiated) tumours and tumours irradiated with 1000 r

and 2000 r in either air or OHP. It is seen that irradiation in O_2 is the more effective. Comparison of the tumour sizes, 14 and 21 days after irradiation in air or OHP, give the following levels of significance—

14 days	1000 r	$t = 4{\cdot}82$	(df = 14)	$p < 0{\cdot}001$
	2000 r	$t = 3{\cdot}70$	(df = 17)	$p = 0{\cdot}002$
21 days	1000 r	$t = 1{\cdot}71$	(df = 14)	ns
	2000 r	$t = 2{\cdot}82$	(df = 14)	$0{\cdot}02 > p > 0{\cdot}01$

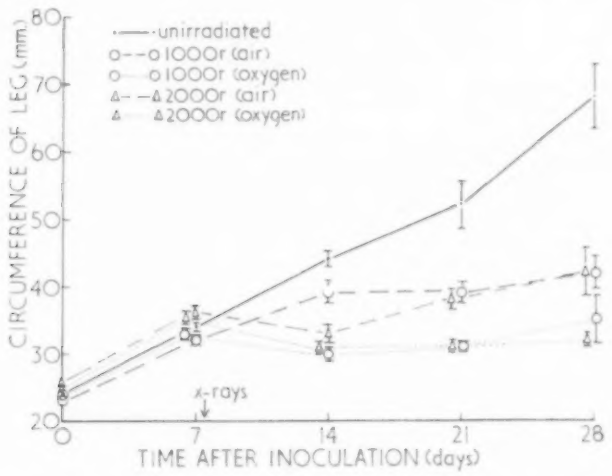


FIG. 4.—Growth curves for control tumours and tumours irradiated with 1000 r or 2000 r in either air or oxygen (45 lb. per square inch gauge pressure). All mice received 400 r whole body X-irradiation 24 hours before inoculation. Vertical lines represent ± one standard error.

However, there is no significant difference in the size of tumours 14 and 21 days after irradiation in air, for 1000 r and 2000 r respectively, nor for these doses received by tumours in OHP. It is considered that tissue reactions complicated the situation and together with other considerations (*vide supra*), suggests that measurements of growth rate of solid tumours *in vivo* is quite unreliable as a quantitative index of radiation effects. Also only a narrow intermediate dose range can be utilised for measuring tumours in limbs of mice to determine modifications of radiosensitivity; lower doses fail to reduce the cell population to sufficiently critical numbers and higher doses cause severe tissue reactions.

(c) *Effect of OHP on reaction of normal tissues.*—At an early stage in this investigation it became apparent that radiosensitivity of normal tissues was appreciably greater as the result of treating animals under high pressure oxygen. Subsequently an attempt was made to classify these reactions, and adopt an arbitrary scale of values to measure degrees of severity (see Methods). The average tissue reaction index x (±SE) is given for tumour doses of 2000, 3000 and 4000 r administered in either air or OHP (Groups I, II and III, Table VI). An extra Group (Ia) was

included in which recipient animals did not receive whole body irradiation prior to inoculation of EAT cells, but the tumours were irradiated with 2000 r either in air or in OHP.

TABLE VI.—*Tissue Reactions in Limbs Expressed as* x *units* ($\pm SE$) *Due to Irradiation of Tumours in Mice Breathing Either Air or Pressurised Oxygen* (45 *lb. Per Square Inch Gauge Pressure ; OHP). All Animals Except Those in Group Ia Received* 400 *r Whole Body Irradiation* 24 *Hours Preceding Inoculation with* 10^6 *Ehrlich Ascites Tumour Cells*

Group	Treatment (number of mice)	Tissue reaction ($x \pm$ SE) in arbitrary units	Radiosensitivity ratio (t)*
I	2000 r in air (10)	1.5 ± 0.2	2·0
	2000 r in OHP (9)	3.0 ± 0.3	
Ia	2000 r in air (10)	$1{\cdot}3 \pm 0{\cdot}2$	2·2
	2000 r in OHP (10)	$2{\cdot}9 \pm 0{\cdot}3$	
II	3000 r in air (10)	$2{\cdot}9 \pm 0{\cdot}4$	1·6
	3000 r in OHP (8)	$4{\cdot}6 \pm 0{\cdot}3$	
III	4000 r in air (9)	$3{\cdot}9 \pm 0{\cdot}4$	1·6
	4000 r in OHP (10)	$6{\cdot}3 \pm 0{\cdot}2$	

* $t = \frac{X_{OHP}}{X_{air}}$. Mean value for Group I, II and III, $t = 1{\cdot}7$.

For all four groups, the tissue reaction (x) in OHP was significantly greater than that in air ($p < 0{\cdot}001$ for comparisons at each dose level). There was no significant difference between air treated animals for Groups I and Ia, nor between OHP treated animals in these groups, suggesting that "immunological attenuation" due to whole body irradiation failed to affect tissue reactions, and that the "oxygen effect" for normal tissues also was independent of the immunological situation for tumour and host. There were significant differences due to effect of dosage in both air treated and OHP treated groups ; in each case $p < 0{\cdot}02$, with the exception of the comparison for 3000 r with 4000 r in air (Groups II and III) for which $p = 0{\cdot}1$.

Calculation of the tissue sensitivity factor (t) for air and OHP

$$\left(t = \frac{X_{OHP}}{X_{air}}\right),$$

for the four groups gave values ranging from 1·6 to 2·2.

The differences in tissue reactions (S) for OHP and air treated limbs at each dose level, gave the following values :—

$$S = (X_{OHP} - X_{air})$$

Group I	$S = 1{\cdot}5 \pm 0{\cdot}36$
Group Ia	$S = 1{\cdot}6 \pm 0{\cdot}36$
Group II	$S = 1{\cdot}7 \pm 0{\cdot}50$
Group III	$S = 2{\cdot}4 \pm 0{\cdot}45$

These values of S for the four groups are not significantly different, and the conclusion is reached that tissue reactions were increased by a factor of approxi-

mately 1·7 by irradiation in high pressure oxygen. Photographs of mice shown in Fig. 7 demonstrate the difference in tumour response and tissue reactions in the legs of mice irradiated in either air or OHP.

Polarographic measurements of tumour and tissue oxygen tension during OHP

To determine the relative effect of OHP on tumour and normal tissues, mice with large Ehrlich's tumours (inoculated limbs measuring 8–9 cm. in circumference) were lightly anaesthetised with pentobarbital sodium. The surface of the tumour was exposed through a small incision and a platinum electrode inserted to a depth of 8 mm. into the tumour. A second platinum electrode was placed beneath the skin of the thigh adjacent to the tumour: the anode was inserted either in a subcutaneous tissue site or in the rectum. The mouse was placed in the pressure chamber and current measurements made with the galvanometer sensitivity adjusted to give a reading of one scale division for the lowest reading electrode. This sensitivity setting was kept constant for the duration of each experiment. Currents were determined for the mouse breathing air, after flushing of tank with pure oxygen, after compression to various tensions of oxygen, and during decompression. Two typical records are illustrated in Fig. 5. The range of the galvanometer scale (100 divisions) was usually too small to record the full range of oxygen currents generated in normal tissues following compression of the animals with the method employed of selecting the sensitivity for each experiment (e.g. Fig. 5*a*). Although the pair of Pt cathodes used for each animal were chosen to give approximately the same values on calibration, the current values recorded are expressed as arbitrary units and only indicate relative changes for normal tissue and tumour in each individual experiment.

Attention is drawn to the following findings:—

(i) Very large variations in the cathode current in mice breathing air were found for the same electrode inserted in different sites in either normal tissues or tumours. This occurred despite the finding that after the electrode was removed from the mouse, its calibration curve remained essentially unaltered.

(ii) Whilst the initial reading (in air) for the tumour was generally lower than that for normal tissue (Fig. 5*a*), sometimes the reverse held (Fig. 5*b*).

(iii) The responsiveness of normal tissue to a change in the partial pressure of oxygen respired, was generally much greater than that of tumour (Fig. 5*a*). The rise in tumour current usually lagged behind that of normal tissue on compression, and often only rose when the tank pressure had reached 30–45 lb. per square inch. On the other hand sometimes the reverse was found and the lag was more marked for normal tissue (Fig. 5*b*).

(iv) The cathode currents in both tumour and normal tissues were often still rising 10 minutes after 60 lb. per square inch oxygen gauge pressure was reached in the tank. Most of the rise in current sometimes developed during the maintenance of this pressure level in the tank.

(v) The maximum current values reached after compression, were almost invariably much higher for normal tissues than tumours. If electrodes were placed in the necrotic centre of a tumour, the current was almost zero with the animal breathing air, and no rise occurred after breathing pure oxygen at 60 lb. per square inch gauge pressure, for as long as 20 minutes.

(vi) After decompression, the cathode current for the tumour fell much more

rapidly, than the corresponding tissue current as is seen for both examples in Fig. 5; the lag which occurred for the fall of current in normal tissue currents was striking.

As already stated, tumours used in these experiments were large, and both macroscopic examination of the tumour after injection of animals with lissamine

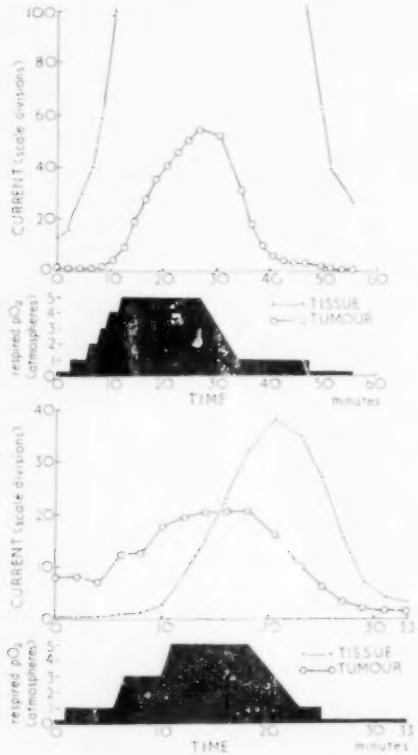


FIG. 5 (*a*, upper and *b*, lower). Cathode current changes corresponding to levels of respired oxygen tension for tumour and subcutaneous tissue in anaesthetised mice. Calibration curves for the pair of cathodes used in each experiment were the same, but the galvanometer sensitivity setting (see text) differs for measurements made in the two animals illustrated. Tip of tumour electrode inserted to a depth 8 mm. below surface of tumour. Advanced tumours approximately 8 cm. in circumference used.

green (Goldacre and Sylvén, 1959) and histological examination, showed the tumour to consist of an outer vascularised zone merging into central necrosis (Fig. 6*a*). In this outer zone most of the tumour tissue was arranged in cords, approximately 80 μ in radius, surrounding capillaries. The periphery of the cord, most distant to the central capillary, merged into necrosis (Fig. 6*b*). The electrode was generally inserted into this outer portion of the tumour consisting of scattered cords amid necrosis, as in Fig. 6*b*. The size of the electrode tip (230 μ) was such as to cause

considerable unavoidable contusion and disruption of tissues and must be recognised in any interpretation of the results.

DISCUSSION

The use of a tumour homograft as a radiobiological indicator is subject to criticisms, which have been stressed on several occasions (e.g. Scott, 1958; Howard Flanders and Scott, 1960). However isogenic systems are not free from similar reservations in regard to strict immunological compatibility, the number of cells inoculated required to constitute a "critical assembly" and the capacity of the tumour stroma and blood supply to keep pace with tumour growth and nutrition. For example, the ED50 values reported for tumour "take" in isologous grafts vary considerably. For C3H mouse mammary carcinoma, Cohen and Cohen (1960) have computed the ED50 for the standard isogenic situation to be 25 cells, using implanted tumour fragments. For the C3H mouse mammary carcinoma, Révész (1958) gives ED50's ranging from $>10^4$ to $<10^7$ cells in different experiments. In the experiments of Suit, Schlachter and Andrews (1960) using C3H/Ba mammary adenocarcinoma in C3H/He mice, 15 per cent of mice did not develop tumours after inoculation with $1{\cdot}8 \times 10^6$ cells, and the ED99 was $\sim 10^7$ cells. For methyl-cholanthrene induced sarcoma in A strain mice, the ED50 was 10^3–10^4 cells (Révész, 1958). For DBA lymphoma the same author reported an approximate 20 per cent tumour incidence for an inoculation of 40 cells; Hewitt (1958) using a CBA lymphatic leukaemia reported ED50's of 1·6, 1·2 and 3·5 cells for intravenous, intraperitoneal and subcutaneous routes respectively.

That strict immunological compatibility does not exist in certain "standard isogenic situations" and that this factor may influence radiosensitivity of the tumour is illustrated by the work of Cohen and Cohen (1960), using the C3H mouse mammary carcinoma isograft, for which immunological attenuation of hosts by whole body irradiation or corticosteroids, reduced the estimated ED50 from 25 to 1 cell, and increased the LD_{50} radiation dose from 5660 to 7500 rads. Conversely "hyperimmunisation" of host mice, increased the ED50 to 200 cells, and reduced the LD_{50} to 4800 rads.

The homologous situation with Ehrlich's ascites tumour varies somewhat with different strains of this tumour and different implantation sites. For the hyperdiploid line used in our experiments, the ED50 for intramuscular inoculation in hybrid mice was 281 (± 71) cells, and reduced to <10 cells by immunological approximation with whole body X-irradiation. In previous experiments, using the same tumour but non-inbred mice of C3H and A origins, the resulting ED50 was not significantly different. By comparison, Warner and James (1959) using the same tumour inoculated in penbred white mice obtained an ED50 of 850 cells for the intraperitoneal route. Donaldson and Mitchell (1959) reported tumour incidences of 100 per cent and 70 per cent for intraperitoneal and subcutaneous routes respectively, after inoculation of Swiss mice with ~ 16 EAT cells. Using a near-tetraploid strain of EAT in non-inbred white mice, Scott (1957) obtained an ED50 of 10–15 cells for the intraperitoneal route; the ED50 (and presumably the homograft reaction) was less for the intramuscular route, but much greater for C57 black strain mice.

In view of these observations, if host-tumour transplantation compatibility is to be based on the smallness of the critical cell number for tumour "take", the

rapidity of its growth, and an absence of spontaneous regressions, there seems little to choose between the majority of standard isogenic situations and a homologous situation after immunological attenuation such as that reported in this paper. Whole body irradiation (400–500 r) caused a substantial reduction in the homograft reaction for EAT, if given 24 hours preceding inoculation, a finding supported by that of Mazurek and Duplan (1959) using a dose 350 r, 24 hours preceding inoculation. Chronic administration of Compound 48/80 depletes endogenous tissue histamine (Feldberg and Talesnik, 1953) and also other amines (Lewis 1958), and was previously found to reduce the heterograft reaction against EAT in rats (van den Brenk and Upfill, 1958). However, no significant reduction in the homograft reaction resulted. This treatment might have been expected to interfere with the immunity reaction in so far as histamine release is generally considered to play a role in antigen-antibody reactions in tissues.

The lethal effect of irradiation on EAT cells *in vivo*, was apparently greatly reduced if whole body irradiated recipient mice were used. Immunological attenuation resulted in an apparent dose reduction by a factor of 3·5, compared with a factor of 1·3 obtained by Cohen and Cohen (1960) for C3H mouse mammary carcinoma isografts. It follows that an overriding influence on radiosensitivity of this order must be reduced to a minimum if other factors affecting radiosensitivity, such as tissue oxygen tension, are to be assessed. In experiments designed to determine the "oxygen effect" the criticism may be raised that local irradiation of the tumours was performed 7–8 days after whole body irradiation of hosts, by which time immunological recovery may have taken place. However, delay of the irradiation-inoculation interval to 7 days was found to increase the ED_{50} to 31 ($\pm$17) EAT cells, which was still considerably less than that for unirradiated hosts. Also, during local irradiation of the tumours, mice received a further whole body dose of X-rays averaging 200 r (see Methods). Nossal and Larkin (1958) have shown that the immunity response remains depressed for at least 9 days in rats after sublethal irradiation with 500 r X-rays.

EXPLANATION OF PLATE.

FIG. 6 (*a*).—Macroscopic appearance of section of untreated solid Ehrlich's tumour removed from thigh and stained with haematoxylin-eosin. Darker areas are viable tumour, pale areas tumour necrosis.

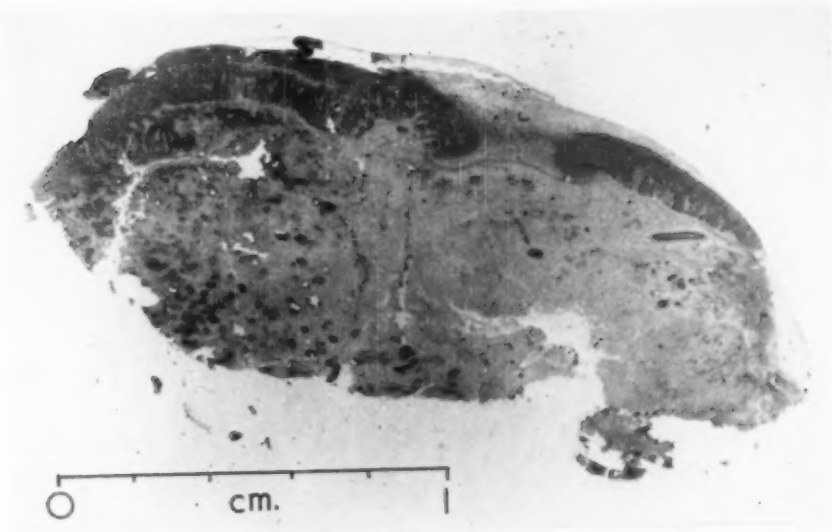
(*b*) Microscopic appearance (×120) of cords of viable tumour cells surrounding capillaries and merging into necrotic tumour. Pyknotic and fragmented tumour cells seen at periphery of cord. Field is selected from central portion of the tumour section in (*a*) and represents the position of a platinum electrode tip in recording changes in oxygen tension (see text).

FIG. 7.—Appearance of tumours in mice treated in air (*a*, *c*, *e*) or in 45 lb. per square inch pressure pure oxygen OHP (*b*, *d*, *f*). In each mouse tumour (T) situated in right thigh.

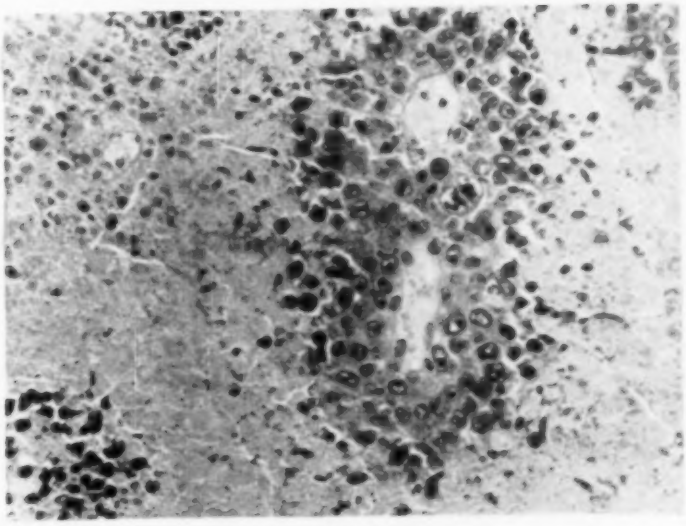
(*a*, *b*) Four weeks after 1000 r X-rays, showing much larger size of residual tumours in air treated mice (*a*), than in OHP treated mice (*b*).

(*c*, *d*) Four weeks after 2000 r X-rays. No residual tumour seen in 3 OHP treated mice (*d*), but residual tumour in the two of 3 air treated mice on right (*c*). Skin reactions are much more severe in OHP treated mice, the treated limbs showing severe moist desquamation and retraction.

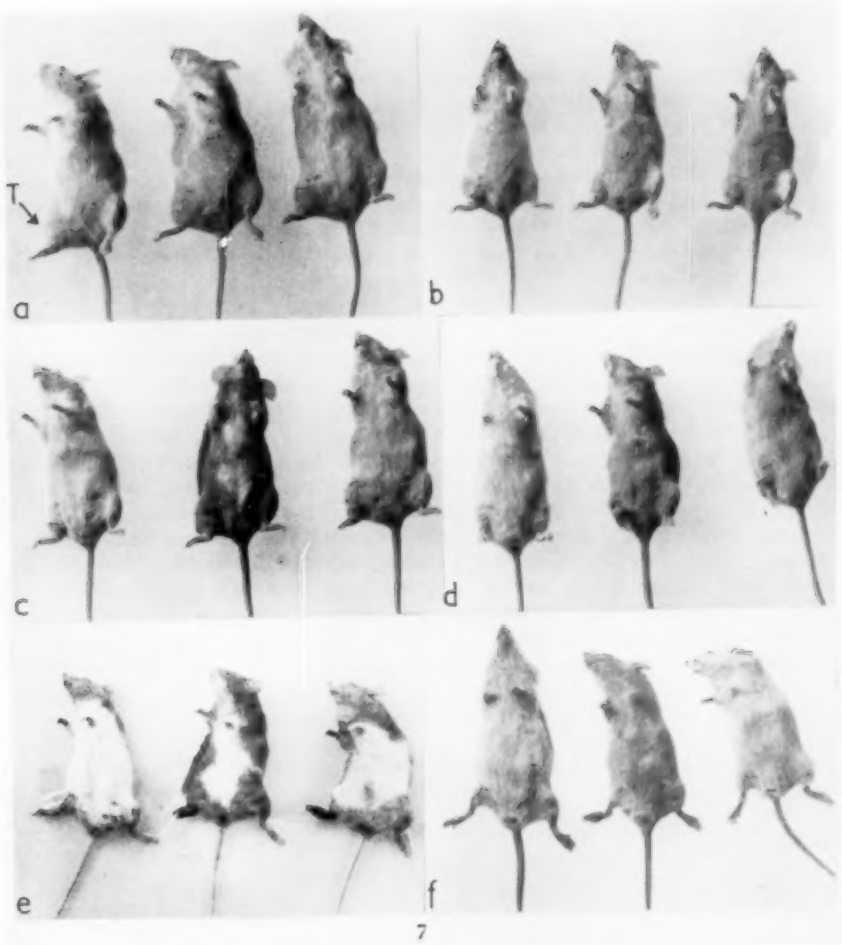
(*e*, *f*) Same 6 mice (2000 r) as (*c*, *d*) but six weeks later. Showing cure of tumours in OHP treated mice (*f*); tissue reactions have subsided and epilation of treated limbs is seen. Two air treated mice on right in (*e*) have large residual tumours invading tissues of abdomen and causing oedema of foot; remaining mouse on left shows no residual tumour; the dose of irradiation (2000 r) has not caused epilation.



6a



6b



7

The studies reported for growth rate of solid tumours resulting from cell inocula of different orders, demonstrated the unreliability of this method in determining the number of cells remaining after a sublethal treatment. The total number of cells which have accumulated at any one time in a solid tumour is largely an index of *tissue form* and a result of the complex interactions of similar and dissimilar cells which go to make up an "organised" tumour. In solid Ehrlich's tumour, after 7–10 days of growth, the major portion of the tumour is necrotic, and the stroma and vasculature is limited in its capacity to meet the nutritional requirements and becomes a major factor in determining the tumour size and its viability. In assessing the radiation doses needed to cope with "small" tumours and "large" tumours, many investigators have made calculations based on the data derived from tissue culture studies. Employing more and more artificial conditions of growth *in vitro*, radiosensitivity curves have been obtained, which may be wrongly interpreted in relation to growth and radiosensitivity *in vivo*. So-called "large" tumours *in vivo*, being largely necrotic, may contain many fewer cells, than estimated from their diameters or weights. Furthermore only a proportion of tumour cells may undergo division *in vivo*; differentiation or natural senescence affects the remainder, in addition to death attributable to endogenous or exogenous factors, which interfere with nutrition. It follows that tumour size, is unsuitable to gauge any one factor affecting radiosensitivity as demonstrated by our own results.

The curves obtained for EAT irradiated *in vivo* in air and pure oxygen (45 lb. per square inch gauge pressure) clearly show an increase in radiosensitivity in OHP with a reduction in the LD_{50} from 4000 r (air) to 1670 r (OHP) i.e. an "oxygen effect" factor of 2·4 for this particular situation. The close correlation of the value of the factor obtained with that of 2·3 for EAT cells irradiated *in vitro* under either complete anoxic or oxygenated conditions (Deschner and Gray, 1959) was surprising, and suggests that the tumours were anoxic during irradiation in air, whilst OHP provided full oxygenation. This suggestion is supported by the finding that OHP also caused a very marked increase in skin reactions (factorial increases ranging from 1·6–2·2).

During the first seven days after inoculation, the limb circumference (C) increased from approximately 2 cm. to 3 cm., and assuming the volume to be spherical in shape, this represents an increase in volume (δv) given by

$$\delta v = \frac{C_1^3 - C_2^3}{6\pi^2} \quad . \quad . \quad . \quad . \quad . \quad . \quad \text{(i)}$$

$$= 32 \text{ mm}^3$$

If a tumour cell be considered to occupy an *effective* volume of 10^{-8} cm.^{3} *in vivo* after allowing for the volume occupied by stroma (Cohen and Cohen, 1960), and assuming that at the time of irradiation all the cells were viable, the volume $\delta v = 32$ mm.^{3} represents an initial tumour cell population (n_0) of $9{\cdot}32 \times 10^8$ cells. The inactivation of tumour cells has been shown to be exponential in the case of HeLa cells *in vitro* (Puck and Marcus, 1956) and CBA mouse lymphatic leukaemia *in vivo* (Hewitt and Wilson, 1959*a*). The relationship is given by

$$\log_e \left(\frac{n}{n_0}\right) = -\frac{D}{D_0} \quad . \quad . \quad . \quad . \quad . \quad \text{(ii)}$$

where n is the number of cells surviving dose D and D_0 is the mean lethal dose (Lea, 1955). For an LD_{50} for EAT of 1670 r in high pressure oxygen and a value of $n = 31$ (± 17) cells obtained for titration in mice 7 days after sublethal whole body irradiation, substitution in equation (ii) gives a value $D_0 = 126$ r (OHP), the corresponding value for mice irradiated in air being $D_0 = 290$ r (air). This value for the inactivation dose in oxygen agrees substantially with that obtained by Puck and Marcus (1956) for HeLa cells *in vitro* ($D_0 = 96$ r; recently adjusted by Morkovin and Feldman (1959) to $D_0 = 139$ rads owing to an error in the original dosimetry); by Elkind (1960) for Chinese hamster ovarian tissue *in vitro* ($D_0 = 128$ rads) and by Hewitt and Wilson (1959*a*, 1959*b*) for CBA mouse leukaemia *in vivo* ($D_0 = 162$ r for ^{60}Co γ-rays). It is considered that this calculated value for D_0 in high pressure oxygen, suggests that in animals anaesthetised with pentobarbital sodium and respiring air at atmospheric pressure, the major portion of the tumour was anoxic during irradiation, despite quite elaborate attempts being made to avoid interference with blood supply of the leg, and the evidence provided by polarography and injection of dye. The very marked increase in skin reactions under OHP supports this conclusion. It is considered unlikely that the anaesthetic alone was responsible for this state of anoxia, in both tumour and skin of animals breathing air. Other investigators who have used animal tumours to determine radiocurability report LD_{50} doses in unanaesthetised animals respiring air, which also seem very high. In C3H mouse mammary carcinoma, Cohen and Cohen (1960) calculated an LD_{50} of 5660 rads for the standard isogenic situation; Suit *et al.* (1960) report a value of 5500 r for the same tumour but could only demonstrate a slight effect of OHP at 30 lb. per square inch gauge pressure on cure rate. However, the tissue reactions reported were not significantly increased, and the method used to immobilise animals strongly suggests that anoxic conditions may have prevailed during irradiation which were not overcome by OHP treatment at 30 lb. pressure. Scott (personal communication) has emphasised the marked influence of apparently minor degrees of vascular compression and stasis on oxygenation of tissues. This was also stressed in the report of Gray (1957). It seems quite clear that local irradiation of animal tumours carried out by retracting a subcutaneous skin tumour away from the body causes severe tumour anoxia. For small animals further factors operate which predispose to tissue anoxia. In mice, oxygen consumption per unit body weight is very high, and the slope of the oxygen saturation curve for mouse blood is very flat in comparison with larger animals (Schmidt-Neilsen and Larimer, 1958). These factors predispose to anoxia, a higher diffusion head being required to adequately oxygenate tissues and particularly those tissues with a high oxygen consumption. Also of importance is the finding of Chase and Hunt (1959) that oxygen diffusing through the surface of skin, may modify radiosensitivity. They found that this applied particularly to damage to the superficial cutaneous appendages (resting hair follicles) and for mice respiring air, damage was reduced by nitrogen flowing over the surface of skin during irradiation. Furthermore, if the flow of blood to the skin was prevented by a vascular clamp to decrease the radiation damage to resting follicles, this effect was reversed if the animal was irradiated in a chamber under 2 atmospheres absolute pressure of pure oxygen and radiosensitivity was restored. Chase and Hunt (1959) also showed that deep barbiturate anaesthesia did not significantly affect hair follicle damage, but they mentioned that " the degree of skin damage is noticeably reduced ". As a result of polarographic measurements of oxygen

tension in skin of rats in relation to radioprotective chemicals and the effect of OHP on this action (Jamieson and van den Brenk, 1960), we have found that barbiturate anaesthesia does cause some reduction in oxygen tension but that respiration of oxygen at 1 atmosphere absolute and higher, readily overcomes any skin anoxia attributable to barbiturates. Tissue anoxia caused by a potent chemical protective agent such as 5-hydroxytryptamine, and even chlorpromazine, is much more resistant in this respect. These findings were confirmed by estimations of arterio-venous oxygen saturations using Van Slyke manometry. Taking all these factors into account, together with other evidence that pure oxygen at 1 atmosphere enhances the skin damage in mice (Wilson, 1959) and that 40 lb. per square inch pressure of pure oxygen causes a marked increase in skin reactions in anaesthetised rats (Moss and Haddy, 1960), it is concluded that the skin and tissues of limbs of mice under experimental conditions is poorly oxygenated to a degree that the radiosensitivity is well below the aerobic level and indeed approaches an anaerobic value. Furthermore, the most striking evidence which supports this conclusion is to be found in the literature of reports of radiation doses given to mouse skin ranging from 4500–8500 r (single application)—doses which clearly exceed tolerance in aerobic conditions. In our own experiments we have found single doses of 4000 r unreliable in its effect on tumours, in so far as " radiation amputation " is not infrequently a result, and tends to exaggerate cure rates (as is seen in Fig. 3 for both air and OHP). For this reason it is considered that high doses of this order which exceed tissue tolerance give an erroneous evaluation of the effects of respired oxygen tension on radiocurability.

The EAT tumour is clearly at a disadvantage in relation to oxygenation. Histological examination shows progressive necrosis with growth, and vessels surrounded by cylinders of surviving tumour tissue (Fig. 6*b*), which measure approximately 80 μ in radius. We have little knowledge concerning the dynamics of blood flow in tumours in relation to capillary oxygen gradients, short circuits (arteriovenous anastomoses) and response to pharmacodynamic agents. Nor do we know the extent to which oxygen is consumed by the tumour *in vivo*, and the role of glycolysis as a metabolic mechanism. The oxygen saturation of venous blood leaving tumours has been reported high in comparison to normal tissues (Bierman, Kelly and Singer, 1952 ; Thomas, 1959) suggesting either the presence of arteriovenous shunts, altered oxygen diffusion characteristics or a reduced oxygen consumption due to a predominantly anaerobic form of metabolism.

The polarographic studies reported in this paper, suggest that this method is of limited value, particularly in providing absolute values of oxygen tension in tissues. However, one of the most serious objections to the use of polarography *in vivo*, is the damage caused by electrodes, however small, to tissues and the pooling of fluid and blood around the electrode tip. This, undoubtedly, explains the lag in response to respired oxygen tensions often encountered in both tumour and normal tissues. In optimal circumstances, virtually no lag occurs, but we have found this to be quite unpredictable for " acute " insertions. For " chronic " insertions (McLaurin, Nichols and Newquist, 1959) the electrode tip doubtless becomes surrounded by a pocket of granulation tissue, which would in itself have altered vascular characteristics, and fail to be representative of the respective organ, tissue or tumour. However, the polarographic studies have clearly shown, that whilst the oxygen current of tumour tissue (semi-necrotic as in Fig. 6) is lower and tends to lag as respired oxygen tensions are raised, OHP at 45–60 lb.

per square inch usually raises this current to levels considerably in excess of the value for normal tissues of an animal breathing air, but not nearly so high as that of normal tissues in the mouse breathing OHP. Furthermore, the more rapid fall of the tumour current during decompression suggests that oxygen consumption at this site is high, and the available oxygen low.

The irradiation of EAT cells in the ascitic form in unanaesthetised mice, and titration of these cells in either irradiated or non-irradiated recipient mice (Tables II and III) may be expected to provide a better model for determination of the inactivation dose (D_0) and the oxygen effect factor. Whilst the data provided in this paper would need to be extended considerably for accurate calculation of the inactivation dose, an approximate estimate of D_0 made for 10^2 and 10^3 cell inocula gives the following values :—

	n_0	n	D_0
Irradiated Recipients	10^2	10	230
	10^3	10	310
Unirradiated Recipients	10^3	250	240

It will be noted that these values agree fairly well with the value for D_0 of 290 r for tumours irradiated in air and for which it has been argued that radiosensitivity approached the anaerobic level. Intraperitoneal measurements of oxygen tension in mouse ascites fluid using the Hersch analyser (Gray, 1959) gave a mean value of 14·6 mm. Hg for animals breathing air, and this value corresponded to a radiosensitivity only 20 per cent higher than the anaerobic value, for radiosensitivity of EAT cells determined *in vitro*. Gray also showed that, by administration of pure oxygen at atmospheric pressure, the radiosensitivity was still only 60 per cent above the anaerobic value. This evidence gives support to the calculations made above and the interpretation that radiosensitivity was determined at approximately anaerobic levels for tumour ascites fluid in unanaesthetised mice breathing air.

SUMMARY

The effect of breathing high pressure oxygen (OHP) at 45 lb. per square inch gauge pressure on radiosensitivity of Ehrlich's tumour (solid form) *in vivo* has been studied. Recipient mice were given 400 r whole body irradiation before inoculation of tumour cells to cause "immunological approximation" and a substantial reduction in the homograft reaction. The effect of irradiation was based on cure of tumours 8 weeks after irradiation. Regression curves show that OHP caused a significant increase in radiosensitivity (radiocurability) with a dosage reduction by a factor of 2·4 (±0·5 SE). Tissue reactions assessed 4 weeks after irradiation were increased in OHP by factors ranging from 1·6 to 2·2. Evidence is provided that in mice breathing air at atmospheric pressure, tissue radiosensitivity approached levels corresponding to minimum (anaerobic) values. The results of polarographic measurements of oxygen tension in normal tissues and tumours in animals breathing air and OHP are described and discussed.

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CARCINOGENIC ACTIVITY OF A SERIES OF REACTIVE LACTONES AND RELATED SUBSTANCES

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ALTHOUGH such a wide variety of chemical substances has been tested for carcinogenic activity, the group of lactones has been largely neglected. Thus, Hartwell (1951) and Shubik and Hartwell (1957) together list 1329 plus 779, a total of 2108, different compounds tested, but very few lactones appear in their comprehensive Survey, and in almost all instances even these have not been tested over any adequate period for their possible carcinogenic action to have been detected.

One of the very few lactones hitherto satisfactorily studied in this respect is β-propiolactone. This vesicant substance was found to be mutagenic in *Neurospora* by Smith and Srb (1951), a finding which led Walpole *et al.* (1954) to study the effect of prolonged subcutaneous injections extending over 13 weeks into rats of β-propiolactone, 2 mg./100 g. body weight, dissolved in arachis oil. Of 12 rats so treated, 9 developed sarcomas at the injection site after 28 to 55 weeks from the start of administration. Roe and Salaman (1955) decided that for the skin of the mouse β-propiolactone was undoubtedly an "initiator" in carcinogenesis in the sense used by Friedewald and Rous (1944) giving tumours rapidly when alternate treatments with croton oil as "promoter" were also given. At first they expressed doubt whether β-propiolactone is itself carcinogenic for the skin of the mouse, but this was apparently due to too short a period of observation, since Roe and Glendenning (1956) later observed tumours after 27–52 weekly paintings of 2·5 per cent β-propiolactone in acetone (papillomata arose in 5 mice of 9 treated, becoming malignant in 2 mice after 40 weeks applications). With higher initial doses, the early ulceration and scarring produced were followed by earlier malignant change (3 carcinomas in 20 mice after 21 weeks). Consequently β-propiolactone must be considered definitely a carcinogenic substance, whether given subcutaneously to rats or by application to the skin in mice. In either case repeated applications are necessary for tumour production; fewer applications than those given above have failed to produce tumours in mice (Salaman, 1959), and this is also shown in the present paper to apply to subcutaneous injections in the rat. This negative result was in fact to be anticipated from the short period of persistence of the agent, due to the ease of breakdown of this unstable lactone ring in the body (cf. Dickens, Jones and Williamson, 1956). This also applies to a varying extent to the other lactones whose carcinogenic properties are described in this paper, all of which were therefore applied repeatedly over long periods, and usually from an oily depot to assist in slow liberation and elimination.

Besides being mutagenic and carcinogenic, β-propiolactone also possesses antibacterial and fungistatic properties (Bernherm and Gale, 1952; Gale, 1953). It has been used for sterilizing plasma (Hartman, LoGrippo and Kelly, 1954) and arterial grafts (Rains *et al.*, 1956) and as a toxoiding agent (Orlans and Jones, 1958). Attention has been drawn by Roe and Salaman (1958) to the possible hazard of using a carcinogenic agent for such purposes, and this aspect is also reinforced by observations in the present paper.

The chemical reactivity of β-propiolactone is very high owing to the presence of the highly strained four-membered lactone ring. A detailed physico-chemical study by Bartlett and Small (1950) confirmed earlier findings that the reaction with water molecules followed a unimolecular type of hydrolysis which was independent of the concentration of added (perchloric) acid. On the other hand, a variety of nucleophilic reagents (acetate, halogen, thiocyanate, thiosulphate and hydroxyl ions, in increasing order of reactivity) caused bimolecular displacement (S_N2 type) reactions with cleavage of the bond between the β-carbon atom and the alcoholic oxygen bond, a mechanism different in type from that of the normal ester hydrolysis observed with the usual γ- and δ-lactones. This special type of reactivity is illustrated, for example, by the fact that whereas in simple aqueous solution β-propiolactone becomes hydrolysed to β-hydroxypropionic acid, in aqueous solutions of ionized salts β-substituted salts of propionic acid are produced, e.g. aqueous sodium chloride solution at room temperature yields primarily sodium β-chloropropionate, together with some reaction-products resulting from the further addition of β-hydroxypropiolactone to the primary product (Gresham *et al.*, 1948).

In fact the reaction rates, as studied by Bartlett and Small (1950), of β-propiolactone with nucleophilic reagents strongly resemble those for the attack of the same reagents upon epichlorohydrin and β-chloroethylethylene sulphonium ions: types of chemical structure which are also associated with carcinogenic properties, as in the chemically closely similar carcinogenic epoxides, ethyleneimines and nitrogen mustards (cf. Walpole *et al.*, 1954). The general chemical background of these and other alkylating reagents in relation to their cytotoxic properties has been extensively reviewed by Ross (1953) and most of his conclusions may reasonably be applied to the cytotoxic and carcinogenic effects of β-propiolactone.

As one example of this type of reactivity, we have studied the interaction of cysteine with β-propiolactone which occurs readily at approximately neutral pH in aqueous solution, and have isolated the product in crystalline form as briefly described by Dickens, Jones and Williamson (1956): in the present paper this compound is shown to be identical with *S*-2-carboxyethyl-L-cysteine, as recently synthesized by a different route by Mamalis, McHale and Green (1960). Consequently the product is now shown to be a thioether, in which the sulphur of the cysteine is bound directly to the β-carbon of the propiolactone with opening of the lactone ring. Similar reactions of the ionized thiol group of cysteine with mustard gas, nitrogen mustards and epoxides are reviewed by Ross (1953).

The property of reacting in this manner with substances such as cysteine is also shown by a range of antibacterial substances. The antibacterial action of β-propiolactone has already been mentioned. Similarly, the penicillins also contain a reactive four-membered ring which is present in this case as a substituted lactam structure: Penicillin also reacts vigorously with cysteine in aqueous solution, whereby it loses its bacteriostatic properties. The chemistry of this

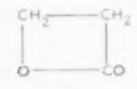
reaction has recently been carefully studied by Nakken, Eldjarn and Pihl (1960) who conclude that the rate-limiting step is a bimolecular reaction involving the mercaptide ion. These authors refer to the widely held view that the antibiotic action of penicillin and its specific binding to sensitive bacteria may involve a reaction with the β-lactam ring. In view of these similarities with β-propiolactone, we have tested penicillin G for its possible carcinogenic activity. Two substituted derivatives of β-propiolactone have also been similarly tested.

It is known that a range of unsaturated gamma and delta lactones also exhibit marked pharmacological activities (for a review see Haynes, 1948). The activities recorded include selective inhibition of the growth of animal tissues, antibiotic activity, inhibition of germination of seeds and of plant growth, and other varied and striking pharmacological effects. Particularly active selective growth inhibitors were certain $\alpha\beta$-unsaturated δ-lactones and a δ-pentenolactone. In some cases a protective influence of alanine and glutathione could be demonstrated (Haynes, 1948), while cysteine (but not cystine) was antagonistic to the δ-hexenolactone. This compound was shown to react with the thiol group, and cysteine counteracted the biological effect of the lactone. It was therefore thought (Haushka, Toennies and Swain, 1945) that this substance exerted its effect on cellular proliferation mainly through its action upon essential thiol groups in the organism affected. A naturally occurring unsaturated lactone probably resembling in structure the above compounds was originally shown to have selective inhibitory effects on the growth in cultures of connective tissue, while permitting free growth of epithelial tissue, by Medawar, Robinson and Robinson (1943). Growth inhibitory effects in young animals, and in Jensen sarcoma in rats, by a factor obtained from malt extract, later tentatively identified by Medawar *et al.* (1943) as the δ-lactone of 5-hydroxyhex-2-enoic acid, had been previously reported by Heaton (1929; see also Medawar, 1937). The long-known dextrorotatory form of this lactone (parasorbic acid) has been isolated and clearly identified from berries of the mountain ash by Kühn and Jerchel (1943) and has been found to exert similar growth-inhibitory properties (Kühn *et al.*, 1943) to those of the synthetic DL-isomer.

From bacteriological studies, a rather closely related series of lactones as well as the above lactone of 5-hydroxyhex-2-enoic acid have been found to exhibit antibiotic properties. Among this series of compounds (Haynes, 1948), substituted $\alpha\beta$-unsaturated lactones, especially those with an unsaturated side chain such as protoanemonin, the γ-lactone of 4-hydroxy-penta-2,4-dienoic acid, obtained originally from *Ranunculus* and *Anemone* species, show marked antibiotic activity. Penicillic acid and patulin are actively antibiotic naturally occurring unsaturated lactones, of which the activity is also abolished by treatment with amino compounds and also with cysteine: patulin is rather toxic: the lethal dose for mice is stated to be 0·2 mg., whereas that for penicillic acid is 7 mg. The reaction of these two antibiotics with cysteine has been studied by Geiger and Conn (1945) who also describe a group of synthetic $\alpha\beta$-unsaturated ketones possessing varying degrees of antibiotic activity: all the active compounds were shown to react with cysteine with loss of the sulphydryl group as shown by the nitroprusside test. Cavallito and Haskell (1945) examined the reaction products obtained when the weakly antibiotic so-called α- and β-angelica lactones (see formulae, Tables I and II) reacted with cysteine and related compounds. The primary reaction was thought to be addition of the thiol group to the double bond

TABLE I.—*Formulae of Compounds Tested for Carcinogenic Activity in the Rat*

(a) Carcinogenic compounds



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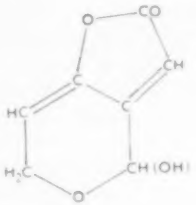

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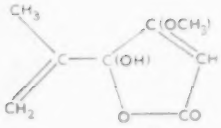

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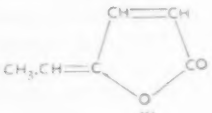

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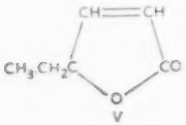

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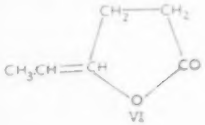

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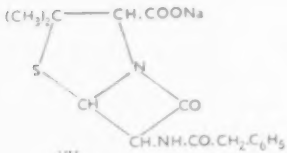

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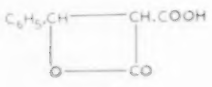

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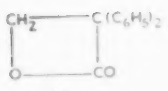

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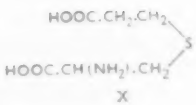
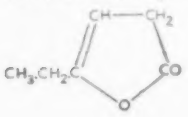


X

(b) Non-carcinogenic



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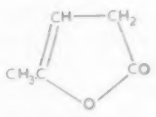

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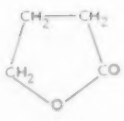


XIII

of the lactones. Saturated lactones (γ-valerolactone) did not react with cysteine, and methylation of the thiol group in cysteine blocked the reaction with angelica lactone. The amino group (e.g. in alanine) did not react directly with these lactones, but the amino group in cysteine did react secondarily after addition of this compound to the double bond in α-angelica lactone, forming a cyclic lactam, which was isolated (Cavallito and Haskell, 1945).

These chemical constitutions and reactivities of the group of biologically active unsaturated lactones have been considered here because they formed the basis of selection of compounds which we have tested for their possible carcinogenic action in the rat. A very wide range of such compounds has been investigated by other workers in connection with their antibacterial action, but an arbitrary selection had to be made from those compounds which were available to us at the time this work began. For comparison, a few less reactive lactones have been included in the series tested. The formulae of the compounds which we have tested for carcinogenic activity are given in Table I.

Nomenclature of lactones used

Because of the confusion introduced by several different systems of nomenclature of lactones, the systematic names corresponding to the formulae of Table I are given in Table II. These are based on the Chemical Society's current usage with the advice of Dr. R. S. Cahn and for brevity the trivial names, also given in Table II, are generally used in this paper.

TABLE II.—*Nomenclature of the Compounds Listed in Table I*

No.	Systematic name	Trivial name used in this paper
I	3-Hydroxypropionic acid lactone	β Propiolactone.
II	—	Patulin (Clavacin).
III	—	Penicillic acid.
IV	4-Hydroxyhexa-2,4-dienoic acid lactone	Methyl protoanemonin.
V	4-Hydroxyhex-2-enoic acid lactone	the 2-hexenoic lactone.
VI	4-Hydroxyhex-4-enoic acid lactone	the 4-hexenoic lactone.
VII	Sodium benzylpenicillinate	Penicillin G (sodium salt).
VIII	2-Carboxy-3-phenyl-3-hydroxypropionic acid lactone	α-Carboxyβ-phenyl-β-propiolactone.
IX	2,2-Diphenyl-3-hydroxypropionic acid lactone	$\alpha\alpha$-Diphenyl-β-propiolactone.
X	*S*-2-Carboxyethyl-L-cysteine	the thioether (condensation product)
XI	4-Hydroxyhex-3-enoic acid lactone	the 3-hexenoic lactone.
XII	4-Hydroxypent-3-enoic acid lactone	α-Angelica lactone.*
XIII	3-Hydroxybutyric acid lactone	γ-Butyrolactone.

* So-called "β-Angelica lactone" is the 4-Hydroxyhex-2-enoic acid lactone, i.e. the double bond is in fact at the α-position, while that in α-angelica lactone is at the β-position, owing to unfortunate historical naming of these compounds.

EXPERIMENTAL

All melting points recorded are uncorrected.

Materials.—β-Propiolactone, α-angelica lactone and γ-butyrolactone were purchased from L. Light and Co. and purified by fractional distillation under diminished pressure.

Patulin (clavacin), m.p. 109–111°, was kindly supplied by Prof. J. H. Birkinshaw, and a second sample of patulin, m.p. 109–110°, was kindly given by Boots

Pure Drug Co., Ltd. Both specimens appeared equally active carcinogenically in the doses used.

Penicillic acid monohydrate, m.p. 62–64° was also a gift from Professor Birkinshaw (Birkinshaw, Oxford and Raistrick, 1936).

Lactones IV (methylprotoanemonin), V (2-hexenoic lactone), VI (4-hexenoic lactone) and XI (3-hexenoic lactone) were generously supplied by the Badische Anilin-und Soda-fabrik A.G., Ludwigshafen, through the kindness of Prof. H. Oettel. The 2- and 3-hexenoic lactones were prepared as described in Beilstein's Handbuch, **17**, 2 *Erg. Werk*, p. 297. Methylprotoanemonin was prepared from dehydrogenating dehydration of β-propionylpropionic acid (DAS 1088,047).

The identity of these lactones was kindly confirmed by Dr. A. E. Kellie in this Institute by measurements of the infra-red absorption spectra.

Penicillin G (crystalline sodium salt of benzyl penicillin, B.P.) containing 1670 international units/mg. was a well known commercial preparation.

β-Phenyl-α-carboxy-β-propiolactone and $\alpha\alpha$-diphenyl-β-propiolactone were given by Imperial Chemical Industries through the courtesy of Dr. A. L. Walpole.

The preparation of *S*-2-carboxyethyl-L-cysteine from β-propiolactone and cysteine is described in this paper. The product, m.p. 210–212° decomp. was recrystallized from water before use. Dr. P. Mamalis of Vitamins Ltd. very kindly provided a comparison specimen of this substance and of its N-benzoyl derivative, and also carried out infra-red absorption spectrum identification on our product.

Animal Experiments

Two-month-old male rats for injection, weighing about 100 g., and 4-week-old female rats of about 50 g. for transplantation experiments were obtained from our own closed breeding colony of animals derived from the Wistar strain. Altogether 295 rats were used.

All substances tested for carcinogenic activity were injected twice weekly into subcutaneous sites in the right flank of the male rats. Repetitive injections into each animal were made as nearly as possible into the same place. When open abscesses developed in any rat as a result of these injections the treatment was withheld from that animal until healing occurred and then resumed as in the rest of the group. Most of the injections were made with a solution of the substance in 0·5 ml. arachis oil, and oil alone was injected into the control rats.

β-Propiolactone was administered in doses of 1 mg. and 0·1 mg. in oil and 2 mg. in water. The latter was a freshly prepared solution for each injection in order to minimize the amount of hydrolysis which occurred before injection. One group of animals was treated with this substance in oil for only 4 weeks, but the treatment of all the other groups with this and other substances was continued until tumours developed, or for as long as our supply of the substances lasted, or for a maximum period of about 60 weeks. The two samples of patulin supplied by Messrs. Boots and Prof. Birkinshaw respectively were tested at doses of 0·2 mg. in oil. Ten times this amount at each injection proved to be lethal to the rats. The 4-hydroxyhexenoic lactone series, which includes the 2-, 3- and 4-hexenoic lactones, and methyl protoanemonin were used at doses of 2 mg. in oil unless rats died, when a lower dose was used. β-phenyl-α-carboxypropiolactone was not freely soluble in oil and, in this case, the saturated oily layer

over an excess of the substance was used for injection. Penicillin G was also insoluble in oil and was injected as a finely ground suspension.

Rats which did not develop tumours within the maximum treatment period were kept under observation until complete absorption of the oil from the site of injection could be demonstrated or the animals died. All rats which survived about 100 weeks were killed at this time and examined post mortem for the presence of abnormalities or tumours.

Suspected tumours were fixed in formol saline for histological study. Small pieces of selected tumours from each group were implanted subcutaneously by trochar into young female rats and their ability to grow recorded over a period of 3 months. Successful transplants were not normally re-implanted.

RESULTS

Tumours developed (Table III) in male rats treated by repeated injections with patulin, β-propiolactone, $\alpha\alpha$-diphenylpropiolactone, β-phenyl-α-carboxypropiolactone, the 2- and 4-hexenoic lactones, penicillic acid, penicillin G, methyl protoanemonin and the condensation product of β-propiolactone with cysteine.

The most effective of these substances as a carcinogenic agent was β-propiolactone which, in oil, gave rise to the appearance of tumours in all the rats treated with a total dose of 5·0 mg. or more over a minimum period of 25 weeks. There was no significant difference in the period required for tumours to appear in groups treated with 1 mg./injection for 29 weeks, with 0·1 mg./injection for 25 weeks or with 2·0 mg. in water/injection for 33 weeks. However, the aqueous solution of this substance was more prone to produce ulceration at the site of injection than the oily solution, making it more difficult to produce uniform exposure of the tissues to its influence. This may be partly responsible for the lower incidence of tumours in this group. β-propiolactone in oil used at a level of 0·2 mg./injection was ineffective when the treatment was only continued for 4 weeks. The compound of this substance with cysteine also produced a significant reduction in its carcinogenic activity, the only tumour produced arising 35 weeks after the completion of 52 weeks' injections in the sole survivor after this long period.

The carcinogenic properties of none of these substances except β-propiolactone have been reported previously.

The great majority of tumours obtained (Table IV) were present at the site of the injection. They were nearly all fibroblastic tumours with varying amounts of collagen formation and were classified as spindle cell sarcomas, fibrosarcomas or myxosarcomas (Fig. 1–6). A large number of the tumours were found to be capable of continued growth as a transplant, and in one instance a tumour obtained by treatment with penicillic acid was maintained as a transplant through twelve generations without marked histological change (cf. Fig. 2 and 6). All the primary tumours showed a sufficient degree of variation in cell size and staining and mitotic activity to justify a histological diagnosis of malignancy, though they did not appear to behave with a high degree of malignancy since for the most part they did not invade the surrounding muscle and subcutaneous tissues, and even massive growths showed no sign of metastasizing. Many of the tumours showed moderate or severe necrosis or calcification when examined.

Penicillin G was found to give rise to two fibrosarcomas at the site of injection, one of which showed a strong ability to grow as a transplant. The rats bearing

TABLE III.—*The Carcinogenic Action of a Series of Compounds Administered Twice Weekly by Subcutaneous Injection to Male Rats*

Substance tested	No. in Tables I and II	Duration of treatment (weeks)	Amount at each injection (in oil unless stated)	Earliest appearance of tumours (weeks)	Number of rats alive at time of tumour appearance*	Number of rats developing tumours	Total period observed (weeks)
Controls-Arachis oil	—	54	0·5 ml.	—	6‡	0	54
		61	—	—	5	0	61
		61	—	107	3	1 (in thorax)	107
β-Propiolactone	I	44	1·0 mg.	29	10‡	10	44
		34	0·1 mg.	25	4	4	34
		33	2·0 mg. (aqueous)	31	4	2	55
		4	0·2 mg.	—	5	0	52
Patulin (Boots)	II	Toxic	2·0 mg.	—	—	—	—
(,,)		61	0·2 mg.	58	4	4	69
(Birkinshaw)		64	0·2 mg.	62	4	2	64
Penicillic acid	III	64	1·0 mg.	48	4	4	67
Methyl protoanemonin	IV	64	2·0 mg.	61	5	3	105
2-Hexenoic lactone	V	64	2·0 mg.	79	4	2	102
4-Hexenoic lactone	VI	58	1·0 mg.	63	5	3	99
		5	2·0 mg.	—	2	0	51
		54	2·0 mg.	93	4	0	93
Penicillin G (Na salt)	VII	46	2·0 mg.	59	4	3†	100
		52	2·0 mg.	84	4	1	105
α-Carboxy-β-phenyl-β-propiolactone	VIII	64	Sat. sol. in oil (<2 mg.)	91	4	1	105
αα-Diphenyl-β-propiolactone	IX	19	1·0 mg.	89	3	1	105
S-2-Carboxyethyl-L-cysteine	X	52	0·5 mg.	87	1	1	87
3-Hexenoic lactone	XI	64	2·0 mg.	—	2	0	105
α-Angelica lactone	XII	61	2·0 mg.	—	5	0	100
γ-Butyrolactone	XIII	61	2·0 mg.	—	5	0	100

* Or at end of observation period, where no tumours developed.

† Tumours included a thyroid alveolar carcinoma and a fibroma at a subcutaneous site remote from the injections and a fibrosarcoma at the injection site.

‡ Groups of 5 rats were used in all experiments except a group of 6 for oil controls and a group of 10 for β-propiolactone.

EXPLANATION OF PLATES

FIG. 1.—Actively proliferating sarcoma from the injection site of a male rat treated with 0·2 mg. patulin in oil twice a week for 62 weeks. This tumour did not grow as a transplant. ×400.

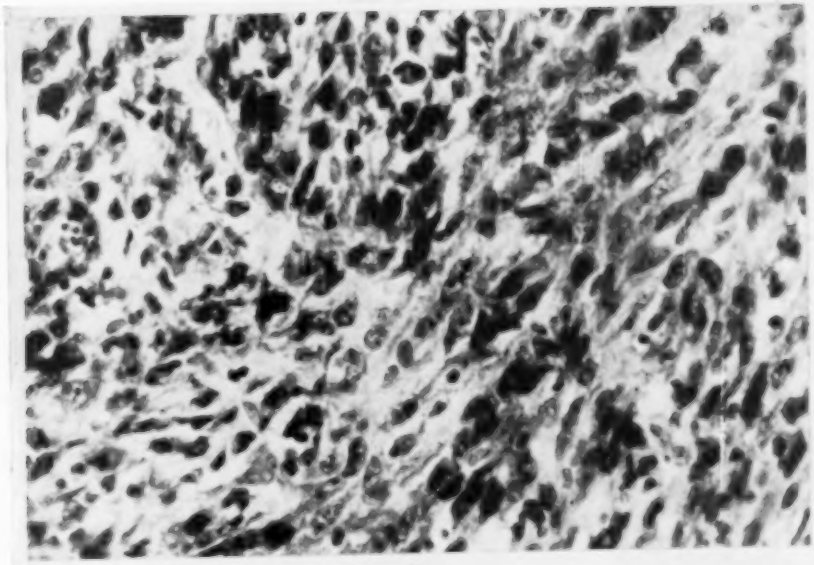
FIG. 2.—Scar tissue with proliferating fibrosarcoma from the injection site of a male rat treated with 1 mg. penicillic acid in oil twice a week for 48 weeks. This tumour grew well in 4 of 5 rats as a transplant. ×400.

FIG. 3.—Actively proliferating fibrosarcoma from the injection site of a male rat treated with 2 mg. methyl protoanemonin in oil twice a week for 64 weeks. The tumour appeared 8 weeks after the injections had stopped and grew well in all of 6 young female rats into which it was transplanted. ×400.

FIG. 4.—Proliferating fibrosarcoma from the injection site of a male rat treated with 2 mg. penicillin G in oil twice a week for 45 weeks. The tumour appeared 27 weeks after the injections had stopped and did not grow as a transplant, unlike the second fibrosarcoma arising in the penicillin series which grew well on transplantation. ×400.

FIG. 5.—Fibroma obtained from the uninjected flank of a male rat treated with 2 mg. penicillin G in oil twice a week for 45 weeks and observed for a further 14 weeks. The rat did not have tumours at the injected site, or elsewhere, when killed. The tumour did not grow as a transplant. ×400.

FIG. 6.—Tumour after 12 successive transplantations of fibrosarcoma obtained from injection site of rat treated with penicillic acid (Fig. 2). This tissue shows an accelerated rate of growth in 100 per cent of the recipients but has not suffered any histological change. ×400.



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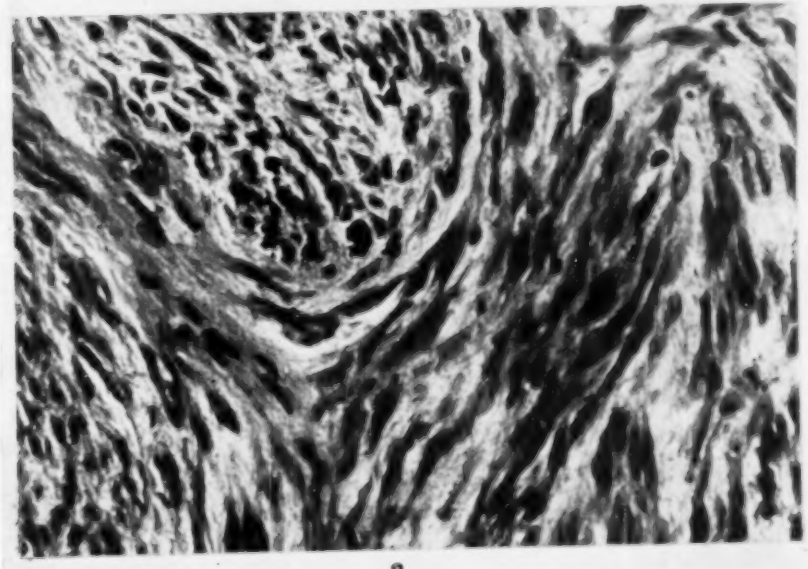

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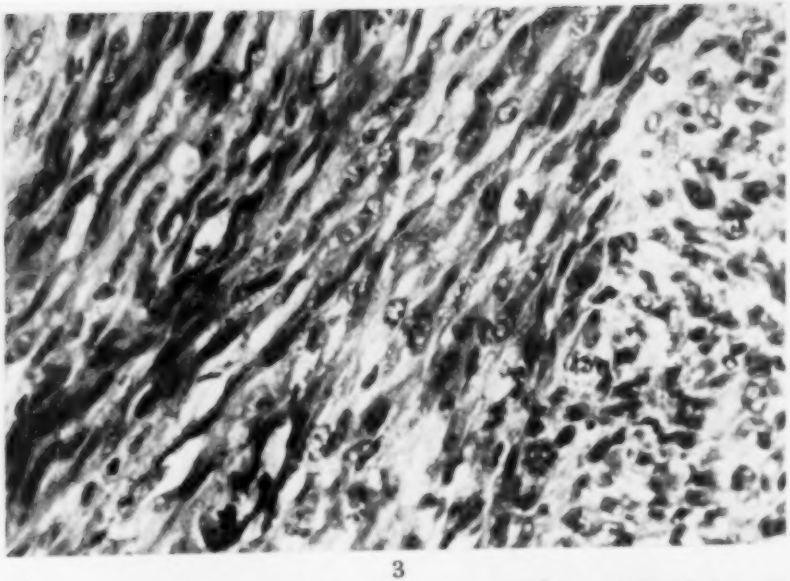
Dickens and Jones.



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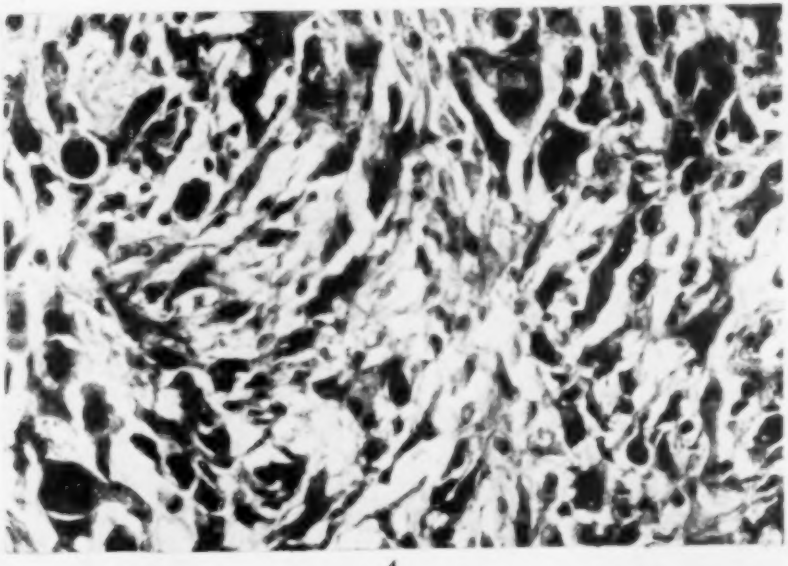

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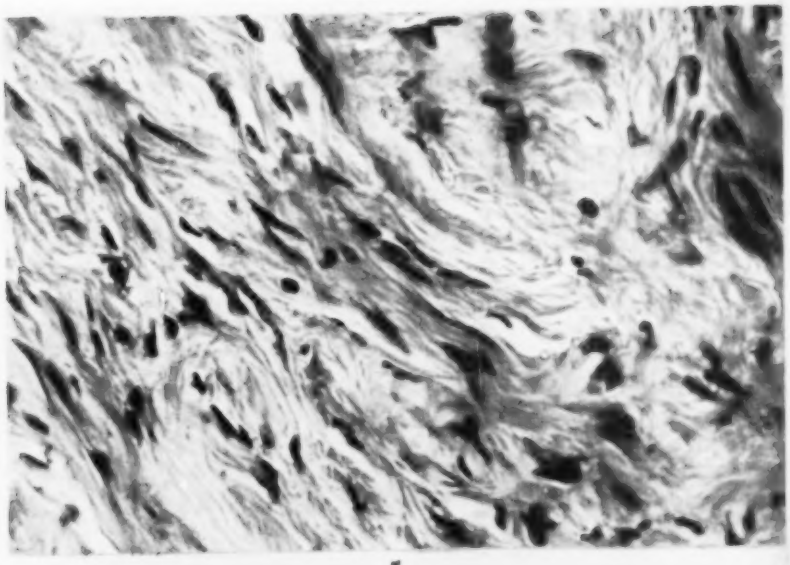

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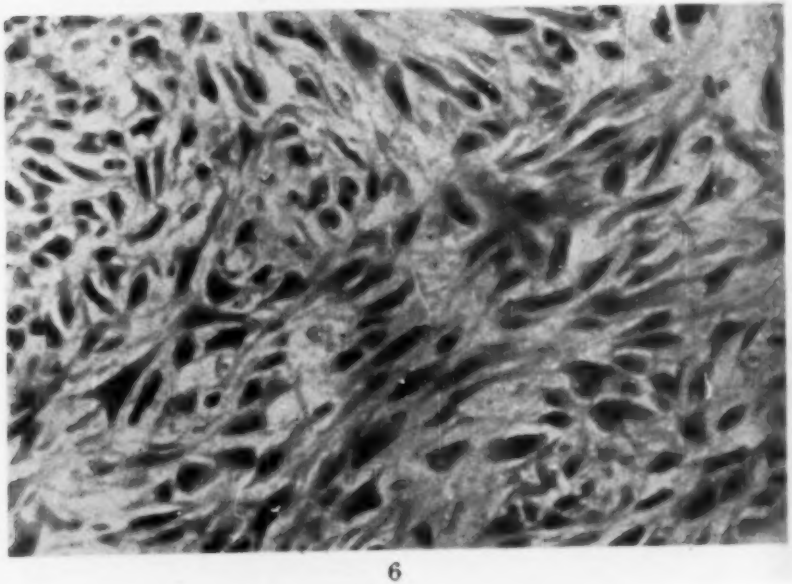


6

these tumours had been given about 200 mg. penicillin (about 330,000 i.u.) over a period of about 50 weeks. Another rat in this treatment group which had received 184 mg. penicillin over a period of 46 weeks developed a large fibroma in the subcutaneous tissues of the flank which had not received the injections. No mitoses were seen in the sections examined and the tumour did not survive transplantation. A fourth rat survived 100 weeks after receiving 184 mg. peni-

TABLE IV.—*Characteristics of Tumours Produced by Male Rats by Repeated Subcutaneous Injections of Lactones and Related Compounds*

Substance injected	Total dose (mg.)	Development time (weeks)	Weight of tumour (g.)	Histology of tumour	Takes in rats of transplants
Arachis oil	(61 ml.)	107	—	Thoracic tumour*	NA
β-Propiolactone (I)	58	29	52	Fibrosarcoma	NA
,,	58	29	?	?	NA
,,	66	33	?	?	NA
,,	70	35	15	?	2/2
,,	70	35	10	?	2/2
,,	68	34	26	?	NA
,,	72	36	54	?	NA
,,	72	36	35	?	NA
,,	76	38	30	?	NA
,,	86	43	50	?	1/2
,,	5	25	9	Fibrosarcoma	NA
,,	6·2	31	20	?	7/12
,,	6·8	34	14	Sarcoma	NA
,,	6·8	34	12	,,	3/10
,, (aqueous)	124	31	8	,,	NA
,, ,,	124	63	50	,,	5/10
Patulin (Boots) (II)	23·2	58	15	Fibrosarcoma	3/12
,, ,,	24·4	62	17	,,	NA
,, ,,	24·4	65	29	,,	NA
,, ,,	24·4	65	11	Sarcoma	NA
,, (Birkinshaw) (II)	24·8	62	10	Fibrosarcoma	0/6
,, ,,	25·6	64	4·5	,,	0/6
Penicillic acid (III)	96	48	3·5	,,	4/5
,,	116	58	21	,,	0/6
,,	128	67	7	,,	6/6
,,	128	67	15	Sarcoma	4/6
Methyl protoanemonin (IV)	244	61	21	Fibrosarcoma	6/6
,,	256	72	33	,,	3/6
,,	256	83	24	,,	0/6
2-Hexenoic lactone (V)	256	79	15	,,	1/7
,,	256	83	7·5	,,	0/6
4-Hexenoic lactone (VI)	116	63	19	Sarcoma	0/6
,,	116	72	13·5	Fibrosarcoma	2/6
,,	116	95	13	Sarcoma	2/6
Penicillin G (VII) (1670 i.u./mg.)	184	59	?	Fibroma*	0/6
ditto	184	72	5·5	Fibrosarcoma	0/6
ditto	208	84	13·5	,,	5/6
ditto	184	100	?	Thyroid carcinoma*	NA
α-Carboxy-β-phenyl-β-propiolactone (VIII)	?	91	13	Fibrosarcoma	5/6
αα-Diphenyl-β-propiolactone (IX)	38	89	13	Myxosarcoma	0/5
S-2-Carboxyethyl-L-cysteine (X)	52	87	41	Sarcoma	1/6

NA—Not attempted.
*—Tumours which developed at sites remote from the injections.

cillin over a period of 46 weeks and at post mortem an enlarged lobe of the thyroid was found. Histological examination showed this to be a carcinoma of the epithelial cells of the thyroid follicles similar to the thyroid tumours which Lindsay, Potter and Chaikoff (1957) have reported to be of spontaneous origin in about 33 per cent of untreated animals examined by them. In contrast to this we have dissected out the thyroids of some hundreds of rats from our colony varying in age from 6 months to well over one year and have never noticed a similar condition. We rarely have the opportunity to examine the thyroids of rats which are 2 years old as was this penicillin treated rat. In this series of experiments, however, over 50 rats were examined which were more than 20 months old and in this selection no other thyroid tumours were found. Whether the thyroid tumour resulted from the penicillin treatment or not it is therefore impossible to say.

There were no tumours found in the oil-injected controls examined at 54 and 61 weeks after the treatment commenced. Among five rats which survived for more than 95 weeks after the oil injections were started there were no tumours at the site of the injections, but one of three rats which survived 107 weeks was found to have a dark red fleshy nodule inside the thoracic cavity, attached to the costal muscle layer, and lying to the right side of the vertebral column at the level of the thoracic vertebrae 5–6–7. This nodule appears to be malignant in that it is not encapsulated and has infiltrated local adipose tissue. Its cells resemble parathyroid tumour, but are not typical of functioning parathyroid cells. Again, a tumour of this type has not been detected in any other rats which have been examined in this laboratory.

Other abnormalities found were : the presence of pyelonephritis in the kidneys of two rats treated respectively with 1 mg. of the 4-hexenoic lactone twice weekly for 58 weeks and 2 mg. methyl protoanemonin twice weekly for 64 weeks. Both these rats were examined after the experiment had continued for about 100 weeks.

One rat treated with 2 mg. of the 3-hexenolactone twice weekly for 64 weeks and examined 41 weeks later showed evidence of peritonitis with adhesions between the intestine and the abdominal wall, extreme bronchiectasis with hardly any of the lungs capable of ventilation, accompanied by the presence of pleural fluid in the thorax. The auricles were much enlarged. Also one adrenal was 3 or 4 times larger than normal with a swollen adrenal artery. Histological examination of this gland showed a picture suggestive of medullary phaeochromocytoma.

Gillman, Gilbert and Spence (1953) report that in rats reared from animals derived from the Wistar Institute phaeochromocytomas were found in 76 per cent females and 85·5 per cent males aged 25 months or more. The incidence in other strains of rats is often not nearly so high, but it is very possible that the phaeochromocytoma found in this study was spontaneous and not related to the treatment with 3-hexenolactone. The same authors report an incidence of 14 thyroid carcinomas in 327 rats aged 13–32 months which further suggests that the thyroid carcinomas also arose spontaneously in our penicillin treated group.

Chemical Properties of the Lactones Used

Condensation of β-propiolactone with cysteine

L-Cysteine hydrochloride (3·14 g., 0·02 moles) was dissolved in 40 ml. water, neutralized to bromothymol blue by sodium hydroxide (9·6 ml. 2 N), and stirred with a solution of β-propiolactone (1·44 g., 0·02 mole, redistilled) in 20 ml. water.

The mixture became acidic and was titrated with 2 N NaOH at frequent intervals to maintain neutrality : 50 per cent of the calculated amount was required after 7 minutes at 20°, and 92 per cent after 2·5 hours, when hydrochloric acid (10 ml. 2 N) was added and the filtered solution was concentrated *in vacuo* until crystals appeared. After leaving at +4° overnight the crystalline compound was collected by filtration, washed with a little water and dried *in vacuo*. Total yield 2·88 g. nearly pure material, m.p. 196° decomp., or 73 per cent of calculated. After one recrystallization from a little hot water, 2·45 g. material (A) m.p. 210–212° uncorrected, decomp., was obtained. Analysis (Weiler and Strauss, Oxford) : Found : C, 37·0 ; H, 5·7 ; N, 7·27 ; S, 16·1 per cent. Calculated for $C_6H_{11}O_4NS$: C, 37·2 ; H, 5·7 ; N, 7·35 ; S, 16·6 per cent. The formula is that of *S*-2-carboxyethyl-L-cysteine, of which a sample (B) kindly supplied by Dr. P. Mamalis (Mamalis *et al.*, 1960) had m.p. (uncorr.) 210–212° (decomp.) ; mixed m.p. unchanged. The N-benzoyl derivative was prepared by treatment with benzoyl chloride in alkali and had m.p. 103–105° (Mamalis *et al.*, 1960, give 106·5–108°), mixed m.p. 103–105°. Analysis (Weiler and Strauss) : Found : C, 52·8 ; H, 5·22 ; N, 4·84 per cent. $C_{13}H_{15}O_5NS$ requires C, 52·8 ; H, 5·1 ; N, 4·7 per cent. The colour value of the unbenzoylated product with ninhydrin, after heating at 100° for 15 minutes, was per mole 90 per cent of that given by pure leucine, indicating one free α-amino group in the molecule. The nitroprusside test for thiol group was negative. On paper chromatography, both before and after oxidation (? to the sulphoxides) with dilute hydrogen peroxide, and whether run in phenol/water or butanol/acetic acid, the R_f values were virtually identical with those obtained with the sample of *S*-2-carboxyethyl-L-cysteine of Mamalis *et al.* (1960) :

	R_f in :	
	Phenol/water	BuOH/acetic
β-Propiolactone product (A)	0·41	0·44
S-Carboxyethyl cysteine (B)	0·41	0·46
Oxidized product (A)	0·27	0·26
Oxidized product (B)	0·27	0·26

We are greatly indebted to Dr. J. K. Whitehead for these measurements and for assistance in identification of our product.

Consequently the reaction of β-propiolactone with cysteine may be written :

$$\mathrm{R.S\!-\!H} + \mathrm{CH_2\!-\!CH_2\!-\!O\!-\!CO} \rightarrow \mathrm{R.S.CH_2\!-\!CH_2\!-\!COOH}$$

where R— is HOOC . $CH(NH_2)$. CH_2— of cysteine, and R . S—H the mercaptide ion.

Rates of hydrolysis of the lactones

The rates of hydrolysis were measured :

(*a*) In bicarbonate buffer at 37° and pH approximately 7 in Warburg manometers in an atmosphere of nitrogen containing 5 per cent carbon dioxide.

(*b*) By titration at 25° with carbon dioxide-free sodium hydroxide and phenolphthalein as indicator.

Rates of reaction of the lactones with aqueous cysteine at approximately neutral reaction

These rates were measured :

(*a*) By mixing, from the side bulb of a Warburg vessel, the lactone (usually 20 μmoles) with a solution of cysteine (20 μmoles) in 0·025 M sodium bicarbonate at 25° under nitrogen/5 per cent CO_2, and measuring the acid production manometrically as the CO_2 evolved.

(*b*) By measurement of free sulphydryl in a mixture, as under (*a*), by means of N-ethyl maleimide (Roberts and Ronser, 1958).

(*c*) By a similar reaction but with the use of the method of Sullivan, Hess and Howard (1942) for cysteine estimation.

This variety of methods was necessary because the lactones variously interfered with the colour reactions and with several of them the nitroprusside test, for example, was brown instead of purple and the results were completely unreliable.

The approximate relative rates of hydrolysis and of reaction with the sulphydryl group of cysteine are summarized in Table V.

TABLE V.—*Rates of Hydrolysis and of Reaction with Cysteine of the Lactones*

	Lactone	Rate of hydrolysis	Rate of reaction with cysteine	
			Acid production	Loss of SH
I	β-Propiolactone	+++[a]	+++[c]	+++
II	Patulin	−	+++	+++[e]
III	Penicillic acid	instantaneous[b]	?[b]	+++[e]
IV	Methyl protoanemonin	±	++	+
V	4-Hexenoic lactone	±	++	++
VI	2-Hexenoic lactone	−	−	+
VII	Penicillin G	±	+++[d]	+
XI	3-Hexenoic lactone	+	+++	±
XII	α-Angelica lactone	±	+++	±
XIII	γ-Butyrolactone	−	−	±

a. k (unimolecular) at 25° : found = $3·9 \times 10^{-3}$ min^{-1}.
b. The lactone is in tautomeric equilibrium with the corresponding ketoacid, 4-keto-3-methoxy-5-methylene-hex-2-enoic acid.
c. k (bimolecular) at 25° : found = 4·6 lit. $mole^{-1}$. min^{-1}.
d. k (bimolecular) at 23° is 4·9 lit. $mole^{-1}$. min^{-1}. (strongly pH-dependent : Nakken *et al.*, 1960).
e. See Geiger and Conn (1945).

DISCUSSION

As far as we are aware only β-propiolactone among the present series of compounds has hitherto been shown to be carcinogenic. In fact, the only other member of this series previously tested appears to be penicillin, of which various derivatives are cited as giving no tumours in the survey by Shubik and Hartwell (1957), where they appear under reference numbers 755, 768, 792, 895 and 910. Most of these penicillin derivatives were given orally, however, the longest administration being orally for 32 weeks in chicks dosed with procaine-penicillin G (Elam, Gee and Couch, 1951) and for 29 weeks in rats with *N*,*N'*-dibenzylethylene-diamine penicillin (Forbes *et al.*, 1953). The longest series of subcutaneous injections cited is only 7 weeks, a period almost certainly inadequate to elicit a carcinogenic response in the rats used, especially as an aqueous solution (of benzyl

penicillin β-diethylaminoethyl ester hydriodide) was injected (Engelbreth-Holm and Roholt, 1953).

In the present series of experiments the control rats given arachis oil did not develop local tumours. The oil was unheated before use, unlike that used for similar control injections into rats by Walpole *et al.* (1954), which was sterilized by heating at 140° C. for 1 hour before use. These authors observed in 91 rats so injected, 14 sarcomas at the injection site after periods of from 60–100 weeks. Whether the difference is related to the pre-heating of the oil or not is unknown. In our control series also, only one tumour at a site remote from the injection was detected.

Local tumours were produced by injection of oily solutions of substances I–X, Tables I–IV. Without tests at higher dilutions it is not possible to attempt more than a very provisional grading of carcinogenic activity, but this would be approximately in descending order down the series from I to X. β-Propiolactone (I) was the most active, in that all survivors at a dosage of 0·1 mg. per injection developed local sarcomas within 34 weeks. It is of interest that even in freshly prepared aqueous solution, in which form it is fairly readily hydrolysed, β-propiolactone was also carcinogenic. Patulin (II) in doses of 0·2 mg. in oil produced local sarcomas in 6 rats among 8 survivors, after a longer period (64–69 weeks). Penicillic acid (III) was not tested in doses below 1 mg., at which level it produced tumours in all animals alive at 67 weeks. The activity of the three lactones, methyl protoanemonin (IV) the 2- and 4-hexenolactones (V and VI) appear to be roughly in that order, with penicillin G (VII) and the two substituted β-propiolactones (VIII and IX) rather less carcinogenically active in the doses given. Finally it was surprising to find one sarcoma in the series of rats injected with doses of 0·5 mg. of the condensation product of β-propiolactone and cysteine, namely *S*-2-carboxyethyl-L-cysteine (X): injected rats dying earlier (47, 52, 69 and 76 weeks after start of injections) had no tumours. We are at present testing β-propiolactone hydrolysed by long standing in aqueous solution at 25° to 3-hydroxypropionic acid, but as yet (45 weeks) no tumours have developed in rats given 1 mg. doses of this compound. Painting β-bromopropionic acid on mouse skin (*Ann. Rep. Brit. Emp. Cancer Campgn*, 1959) gave no tumours either. There is perhaps a possibility that the carboxyethyl radicle may become transferred in the body from compound (X), a point that requires investigation.

Closely related lactones which have not produced tumours in this series are the saturated compound γ-butyrolactone (XIII) and two lactones both having the double bond in the β-position, namely the 3-hexenolactone (XI) and α-angelica lactone (XII).

As a result of this work, the types of chemical structures shown in Table VI have been provisionally allocated potentially carcinogenic or non-carcinogenic properties:

The Roman figures under the formulae refer to the numbers given to the representative lactones in this paper, while the capital letters refer to the group-formulae in Table VI.

The 4-membered rings of the propiolactones (A) or of penicillin (B); and also the various hexenolactones (C, D and E) can be carcinogenic, but not apparently the saturated 5-membered lactone (F) nor the β-unsaturated lactones (G).

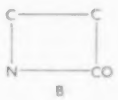
The carcinogenic activity in these series bears no obvious relationship to the rate of hydrolysis (Table V), but there may be a tentative indication of a cor-

TABLE VI.—*Basic Types of Chemical Structure in Relation to Carcinogenicity*

(a) Carcinogenic

A

Compounds I, VIII, IX



B

VII

C

Compounds II, III, IV, V

D

II, IV

E

VI

(b) Not carcinogenic

F

Compounds XIII

G

XI, XII

relation with the reactivity with the cysteine sulphydryl group, though apparently not with the extent of acid production occurring on reaction with cysteine (Table V). It would appear desirable to make a more detailed study of the mechanism of reaction of all these lactones with nucleophilic reagents such as cysteine, since the overall reactions clearly belong to more than one fundamental type. It is worth noting that among the five-membered lactones, the possibility of conjugation of the carbonyl double bond with other double bonds already present in the molecule (lactones II, III, IV, VI) or perhaps readily produced by dehydrogenation (for example of lactone V, giving the doubly conjugated lactone IV), appears to be associated with carcinogenic activity in this series of compounds.

A very wide range of such lactones is available, and many of them have been found to be naturally occurring in plants, bacteria and animals (see Haynes, 1948). Although all the compounds found to be carcinogenic in this paper are weakly so, relative to such highly active carcinogens as benzopyrene, Kennaway (1954) and others have stressed the importance in relation to human cancer which materials of low potency might have if they were to be either formed in animal metabolism or taken up from external sources over long periods.

The group of lactones and lactams of the type described in this paper need much further study with a view to developing the relationship between chemical structure and carcinogenesis in this series, which seems to be emerging. This should be supplemented by a study of the effects of the lactones as antibiotics, on mutations, as alkylating agents, and as selective growth inhibitors, properties which as we have already seen are known to be possessed by some of their members.

SUMMARY

1. Starting from the known carcinogenic property of β-propiolactone, a study has been made of the effect of twice weekly subcutaneous injection into rats of a number of chemically and pharmacologically active lactones and related substances.

2. β-Propiolactone given as a solution in arachis oil was found to produce sarcomas at the site of injection in all rats surviving at 34 weeks at a dose level of 0·1 mg.—about one twentieth of that previously tested. Tested at the 2 mg. dose level the freshly prepared aqueous solution was also carcinogenic. Two substituted β-propiolactones were weakly carcinogenic in the doses used.

3. The antibiotics patulin (clavacin) and penicillic acid were carcinogenic in all surviving rats when tested in 0·2 mg. and 2 mg. doses respectively.

4. Penicillin G (2 mg. doses as the sodium salt) suspended in arachis oil produced one local sarcoma in each of two separate groups of five rats.

5. Arachis oil, unheated, gave no local tumours in 20 control rats, which showed only one tumour (thoracic) at autopsy.

6. Three synthetic unsaturated 5-membered lactones (methyl protoanemonin, 2-hexenoic acid lactone and 4-hexenoic acid lactone), in doses of 1 or 2 mg., produced local sarcomas on injection.

7. Attention is drawn to the properties of antibiotic action and of growth inhibition, and their inactivation by reaction with cysteine, shown by certain of this group of carcinogenic agents. The interaction of β-propiolactone and cysteine has been studied and the product isolated and identified as *S*-2-carboxyethyl-L-cysteine. This substance itself had only very weak carcinogenic properties.

8. Two main types of chemical constitution appear associated with carcinogenic activity in this series: a highly strained 4-membered ring (as in the β-propiolactones and the lactam ring in penicillin) or a lactone ring having double bonds at the 2 or 4 position, or preferably at both. If there was no double bond, or if it was at the 3-position, the lactones tested were inactive.

We would like to express our gratitude to Dr. A. C. Thackray for his kindness in giving opinions on all the sections examined histologically, and also to Professor I. Doniach for advice on the thyroid and (?)parathyroid tumours. Gifts of materials from Professor J. H. Birkinshaw, Dr. P. Mamalis, Professor H. Oettel and Dr. A. L. Walpole are gratefully acknowledged. Dr. J. K. Whitehead and Dr. A. E. Kellie and Mr. D. H. Williamson kindly provided chemical advice and help, and technical assistance was provided by Mr. S. Graves, Mr. E. D. Decker, Miss Frances Bell and Miss Judith Cooke.

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THE EFFECT OF DIESEL-OIL SOOT ON DIFFERENTIATION OF THE CULTIVATED MUSHROOM: *AGARICUS CAMPESTRIS* VAR. *BISPORUS*

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A STRIKING tumour-like upset in morphology, named "rose-comb" disease (Lambert, 1930), was found in a commercial crop of mushrooms at Aberystwyth (Thomas, 1951). Studies on the effect of external agents on differentiation during fruit-body development, carried out at Aberystwyth, conclusively showed that "rose-comb" disease was due to the effect of tarry fumes from coal-coke mixtures or mineral oil used for heating mushroom houses. The disease was produced experimentally under controlled conditions, and the tumorous growths involved were shown to manifest cytological changes similar to those found in tumours of higher animals (Thomas and Evans, 1954; Evans, 1955).

Coal-tar and mineral oils have been known to be carcinogenic for a long time. Coal tar was used by Yamagiwa and Ichikawa (1916) for the first experimental production of cancer by chemical treatment. Later Komuro (1931) demonstrated that coal-tar was also carcinogenic for plants. Then Kennaway and Heiger (1930) prepared the first chemically pure hydrocarbons, and identified benzopyrene as the cancer-producing agent in soot, mineral-oil, pitch, and coal-tar opening a valuable line of experimental research on the chemical nature of carcinogens. In humans, however, evidence on carcinogenic activity most closely akin to experimental data is obtained by studying cases of occupational cancer. Thus Henry (1947) has attributed certain occupational cancers to the action of creosotes or mineral-oil on human skin.

However the tumours produced on the mushrooms, like all plant tumours, were not considered cancerous because no invasive behaviour was observed. It was concluded that the mushroom tumours were analogous to the benign tumours of animals.

Evans (1955) found that tumours of the cultivated mushroom could be induced by the action of coal tar fumes, Diesel-oil vapour and the vapours of a proprietary compound containing tar acids. Tumours were only produced when these substances were applied in the vapour form, and direct application was frequently lethal. In addition, non-tumorous abnormal differentiation was produced by the proprietary compound containing tar acids and phenol. Failure to produce any effect by means of certain pure non-volatile carcinogenic hydrocarbons (20-methylcholanthrene, 3,4-benzopyrene, 1,2,5,6-dibenzanthracene, anthracene, pyrene and phenanthrene) was attributed to the failure to find a suitable solvent for these substances that was not also toxic to the fungus. It was concluded that no comparative assessment of the different substances in producing tumours would be possible until some of the technical difficulties had been overcome.

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The present study was concerned with determining whether a non-volatile product of Diesel-oil combustion, a soot, would be carcinogenic for mushroom tissue, and if possible to provide tumours for further study of the nature of the change involved in tumour formation. In order to do this, technical difficulties previously encountered in attempting to induce tumours by means of carcinogens not volatile at temperatures of mushroom culture, would have to be overcome.

An active animal carcinogen, benzopyrene, was isolated by Lindsay and Cooper (1951), Kotin, Falk and Thomas (1955) and Lyons (1955) from soot scraped from Diesel-oil-engine exhaust pipes. The carcinogenic activity of similar soot was demonstrated by Beck (1955) who produced lung tumours of mice by injecting a suspension of soot.

MATERIALS AND METHODS

(i) *Genetic constitution*

The cultivated mushroom unlike the majority of hymenomycetes is *homothallic* —normal fruit bodies are formed from single spore cultures (Lambert, 1929; Klioushnikova, 1938; Kligman, 1943). Klioushnikova (1939)* concluded that "The homothallism of the cultivated mushroom has as its basis a characteristic distribution in the spores of 4 haploid nuclei from the basidium that have various sexual features (heterokariotic bisexuality): each spore containing 2 non-sister nuclei, at the same time." This conclusion was supported by the cytological studies of Evans (1955), who found that there was a relationship between the alignment of the spindles at the second division of meiosis and the percentage of fertile spores found by Sinden (1937) and Kligman (1943). There appeared to be a selection for a type of alignment which ensured a high percentage of heterokaryotic (fertile) basidiospores.

With regard to the genetic stability of the *homothallic heterokaryotic state*, Kligman (1943) found in his genetic studies that "Mycelium derived from the multisporous method or by tissue culture technique do not ordinarily exhibit variations from the parent."

An abnormal fructification was found by Walcot (cited by Kligman, 1943). It arose from a single spore culture and failed to differentiate into stipe or pileus and lacked hymenial tissue. A similar abnormality arose in a pot treated with Diesel-oil vapour by Evans (1955) who suggested that its form was either "a result of a particular combination of nuclear types in the mycelium or from mutation." Normally, migration of nuclei occurs through conjugation tubes between hyphae of the mycelium—a possible mechanism for maintaining an even distribution of genetically different nuclei, which usually only segregate at spore formation.

It is possible that tumours also arise spontaneously. Atkins (1951) commented that "the fact that crops produced isolated examples of rose-comb, suggests that the presence of mineral oil is not the sole factor causing this abnormality."

However no abnormal differentiation of untreated fruit bodies has occurred in the variety used for both the experiments of Evans (1955) and the present investigation: the "White" variety of Messrs. Darlington and Sons, Worthing, England. The spawn of this variety was propagated vegetatively in pure culture under aseptic conditions, so that the desired qualities of the strain were main-

* This paper was kindly translated from Russian into English by Dr. R. N. Rast, U.C.W., Aberystwyth.

tained uniformly in the same way as vegetatively propagated horticultural plants.

Mycelium propagated in this way may be regarded as a clone of cells of identical genetical constitution.

(ii) *Controlled conditions for growth*

The mushroom was grown in a standard synthetic compost (in 9 in. plastic pots), prepared, distributed and inoculated with spawn as described by Evans (1955).

Pots containing inoculated compost were placed in a room where the temperature was thermostatically controlled at 73° F. and the relative humidity at 90 per cent to create optimum conditions for mycelium growth, which took about three weeks to permeate the compost.

Then the compost was covered with a 1-in. layer of medium loam subsoil (sterilised by autoclaving at one atmosphere for half an hour) with its pH adjusted from 5 to 7 by adding ground limestone. Regular watering of this casing soil was commenced to keep it moist.

In order to promote fruit-body production, at this stage, the temperature and humidity were adjusted to the optimum for fruiting : 59° F. and 70–80 per cent relative humidity. Also the air in the room was renewed each day since ventilation was important at this time. Young fruit bodies first appeared about two weeks after casing the compost. The stages in fruit body development to be referred to are :

Pinhead stage : fructification up to 1 cm. in diameter.

Button stage : fructification greater than 1 cm. in diameter, and with the veil intact.

Mature stage : fructification in which the veil has ruptured, and the gills are exposed.

Most of the pinhead initials were formed at the surface between the compost and the casing soil or the lower half of the casing soil, so that the fruit bodies pushing through the surface of the casing soil were generally at the late pinhead or early button stages. It took ten to fourteen days for pinheads to reach maturity.

(iii) *Treatments with soot*

The soot obtained was a fine black powder, insoluble in water and olive oil (the only solvent for non-volatile carcinogens which was not toxic to the mushroom). It was decided to apply the soot dry in several ways, since the humidity of the room and the watering of the pots would soon render it moist. Then it was hoped that possibly the products of micro-organisms or mushroom cells of either mycelium or fruit body might render some constituents soluble and then absorbable into the mushroom cells.

In order to distinguish the effect of substances other than carbon in the soot, all methods were replicated using finely ground animal charcoal, in the following sequence.

(a) *Mycelium growing in pure culture on malt-agar plates* (4 per cent malt, 2 per cent agar, pH 7).—Soot was applied at several points around the growing

edge of the mycelium radiating from the centrally placed inoculum, in order to test possible toxicity.

(b) *Young fruit bodies at the surface of the casing soil.*—It was necessary to treat all fruit bodies in each pot, or only untreated ones would develop, because usually only from four to eight fruit bodies developed to maturity after competition between up to twenty pinheads differentiating in each pot over a period of ten days.

Soot was sprinkled uniformly over the surface of the pinheads and pilei of the buttons.

(c) *Early pinhead stage, within the casing soil.*—In order to treat this stage, soot was included in the casing soil, because pinhead initials were usually formed at the surface between the casing soil and the compost or in the casing soil. Soot was included in the casing soil at concentrations ranging from 1 to 10 g. soot in 100 g. soil. After adding soot, the casing soil was adjusted to pH 7 by adding ground limestone.

(iv) *Techniques for cytology, histochemistry and growth measurement*

Material for squash preparations was fixed in chloroform, alcohol, acetic acid, in the ratio of 7 : 5 : 2—a modified Carnoy's solution (Evans, 1955), the increase in the chloroform fraction facilitating removal of fatty substances present in the cytoplasm. Squashes were stained with aceto-carmine, mordanted with iron acetate.

For sectioning, material was fixed in Randolph's fixative (cited by Darlington and La Cour, 1947) or in acetic alcohol in the ratio 1 : 3 and stained with the basic dyes : azure B, toluidine blue, methyl-green-pyronin Y (Swift, 1955), or a saturated phenyl-fluorone solution after hydrolysis (Turchini, Castel and van Kien, 1948 ; Glick, 1949), primarily for indicating the distribution of ribonucleic acid (RNA).

The interpretation of the staining reactions with phenyl-fluorone and the basic dyes for RNA was made accurate by treating control sections with ribonuclease to remove RNA specifically before staining (Swift, 1955).

The growth rate of normal and tumour tissue in culture was measured by the Petri dish method (Tomkins, 1932). Measurements were made of the increase in diameter of standard size inocula cut from the margin of tissue cultures by means of a sterilised cork borer.

EXPERIMENTAL RESULTS

(i) *Tumour induction*

Soot applied to the margin of mycelium growth in pure culture on agar had no effect on growth (Fig. 2).

Out of a series of treatments involving 140 pots, tumours (Fig. 1) were only produced when soot was included in the casing soil, at a concentration of 5 g. per 100 g. casing soil. Lower concentration produced no tumours and higher concentration produced no mushrooms. Mushrooms also failed to develop in control pots with more than 5 g. animal charcoal per 100 g. soil, suggesting that failure of fruit body formation in pots treated with more than 5 g. soot was due to physical impediment of aeration, rather than a chemical effect (since charcoal consists almost entirely of pure carbon, which is chemically inert).

Morphologically the soot induced tumours were similar to some produced previously by Diesel-oil vapour treatment : both in shape and in having no re-differentiation of gill tissue on their surface (Evans, 1955).

(ii) *Cytological study*

The types of cell encountered are represented by the letters N for normal cells and T for tumour cells. The numbers 1 and 2 after the letters N and T indicate the non-vacuolated and vacuolated condition of the cells respectively.

As far as could be ascertained from squash preparations the types of cell were similar to those due to Diesel-oil vapour described by Evans (1955) : basophilic non-vacuolated cells with actively dividing nuclei (T1 cells), with intermediate stages to less basophilic vacuolated cells containing resting nuclei (T2 cells). However unlike the Diesel-oil tumours, a comparison of the average number of nuclei per cell of the soot induced T2 cells with N2 cells of the same fruit body showed no increase. Also none of the abnormal nuclear activities found in Diesel-oil tumours (such as endomitosis, multipolar spindles, chromosomes aggregation, polyploidy, aneuploidy or micronuclei) were found in the soot induced tumours.

(iii) *Histochemical distribution of RNA*

A study of the RNA distribution was made since the RNA content of a tissue indicates the metabolically active cells because RNA content is closely correlated with the level of protein synthesis (Caspersson, 1950 ; Brachet, 1950 ; Gale and Folkes, 1954).

Sections were stained to reveal the RNA distribution in soot induced tumour tissue and compared with that during normal differentiation of the fruit body, paying particular attention to the pinhead stage since this was the stage subjected to soot treatment.

Fig. 3 shows the RNA distribution, as indicated by toludine blue staining in four stages during pinhead differentiation. The same pattern of RNA distribution was indicated by azure B, pyronin Y, and phenyl-fluorone. The highest intensity of RNA has a marginal distribution in stage (a) and apical distribution in stages (b) and (c) as the pileus develops.

The highest intensity of RNA within the apical region of the pinhead is in the developing gill tissue and the rim of the developing pileus. (The highest RNA intensity coincided with the parts of the pinhead where divisions could be found in squash preparations.)

In tumour tissue the intensity of RNA distribution resembled the apical and marginal distribution in an undifferentiated pinhead (cf. Fig. 3 and 4).

Like the young pinhead, the cells with highest RNA content (N1 and T1 cells) occupied the marginal and apical zones, while the vacuolated cells, containing less RNA (N2 and T2 cells), occupied the inner zone or trauma. On the other hand, a comparison of the RNA content of T2 and N2 cells within the same fruit body, at maturity, showed that the RNA content of T2 cells was higher than the N2 cells (Fig. 4, cf. a2 and c1). Nevertheless the highest RNA content of all was found in normal tissue : the hymenium. Even in the young pinhead the developing gill tissue contained more RNA than other N1 cells (Fig. 3). Then at maturity RNA content was higher in the hymenial tissue than in the T1 and T2 cells of the same fruit body.

(iv) *Growth measurement*

A comparison of the growth rate of eight tissue cultures with the same number of tissue from the same pileus as the tumour tissue, showed no difference.

DISCUSSION AND CONCLUSIONS

In order to determine how the tumours arose it is illuminating to examine the normal pattern of differentiation in the fruit body, for comparison with the pattern in tumours.

The general outline of morphogenesis was described in terms of changes in cell shape and size during the formation of the primitive tissues, by Atkinson (1906), Hein (1930), Corner (1935) and Gaumann (1952). All the zones of cell types that would develop into the mature tissues, could be distinguished by the end of the pinhead stage (Fig. 3c). Since the pileus develops endogenously the type of fruit body development was described as hemangiocarpous, unlike that of *Collybia velutipes* where the pileus development is exogenous and the fruit body described as gymnocarpous (Corner, 1935; Gaumann, 1952). Gill tissue developing endogenously does not become exposed until maturity, whereas gill of an endogenous pileus is exposed during development (Fig. 5).

Another aspect of morphogenesis was studied by Bonner, Kane and Levey (1956). They examined the mechanics of growth of the cultivated mushroom by measuring the surface of the fruit body during development and some corresponding changes in histology seen in sections. Growth zones were identified by following the increase in width between equidistant carmine spots painted on the surface of the fruit body at a very early stage. In the stipe most growth occurred in the region next to the cap. In the pileus growth was also uneven, being fastest at the edge and gradually slower at the centre. After the 15–20 mm. stage (in height) in the stalk there was little or no increase in the number of filaments and cells ceased dividing. From the 15–20 mm. stage onwards comparison of dry and wet weights showed that further growth of the fruit body could be attributed to elongation of cells since there was a logarithmic increase in dry matter.

On another level of observation, fundamental cytological studies by Maire (1902), Sass (1928), Colson (1935), and Sarazin (1938) were primarily concerned with the development of the four nuclei resulting from meiosis in the basidium. Evans (1955) paid more attention to the behaviour of nuclei in somatic tissue. Cells undergoing active mitotic activity were most prevalent at the pinhead

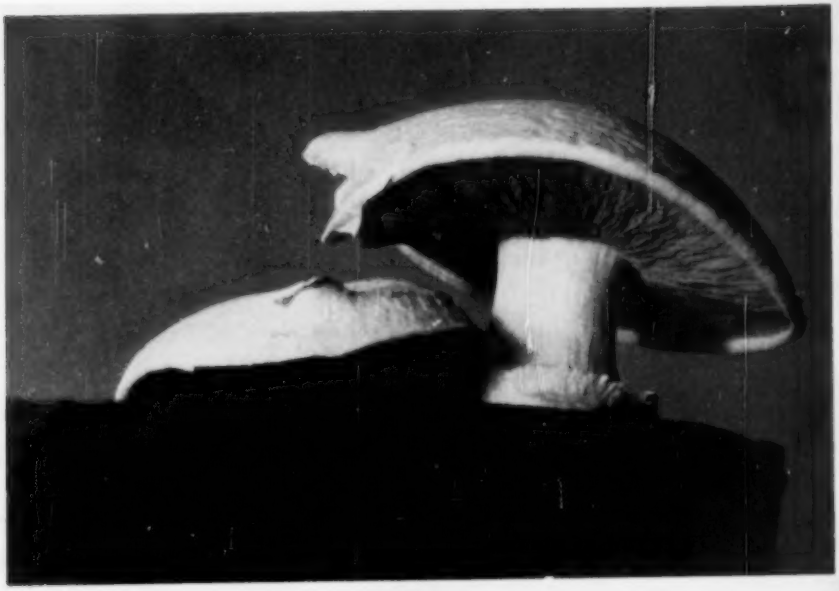
EXPLANATION OF PLATES

FIG. 1.—Tumours of the pileus induced by Diesel-oil soot treatment of young fruit bodies at pinhead stage.

FIG. 2.—A test for toxicity; mycelial growth uninhibited by Diesel-oil soot placed at three points around the margin of normal mycelium growing in pure culture on malt agar.

FIG. 3.—Sections through normal fruit bodies at several stages during pinhead differentiation, stained to show the distribution of ribonucleic acid (RNA).

FIG. 4.—Sections through tumour tissue and normal tissue in the mature pileus (Fig. 1) to show the relative distribution of ribonucleic acid (RNA). a: L.S. tumour tip, a1 and a2 showing different staining intensity; b: T.S. tumour, b1 is part of T.S. enlarged; c: L.S. tumour base, c1 is part of L.S. normal pileus enlarged; h: L.S. gill tissue of mature pileus (the photograph shows part of L.S. one gill lamella).



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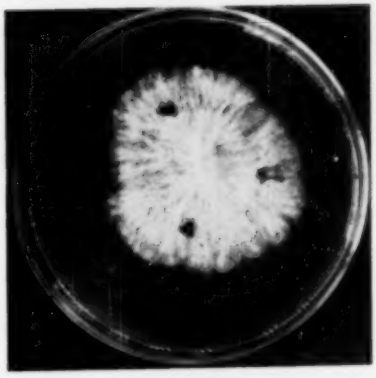

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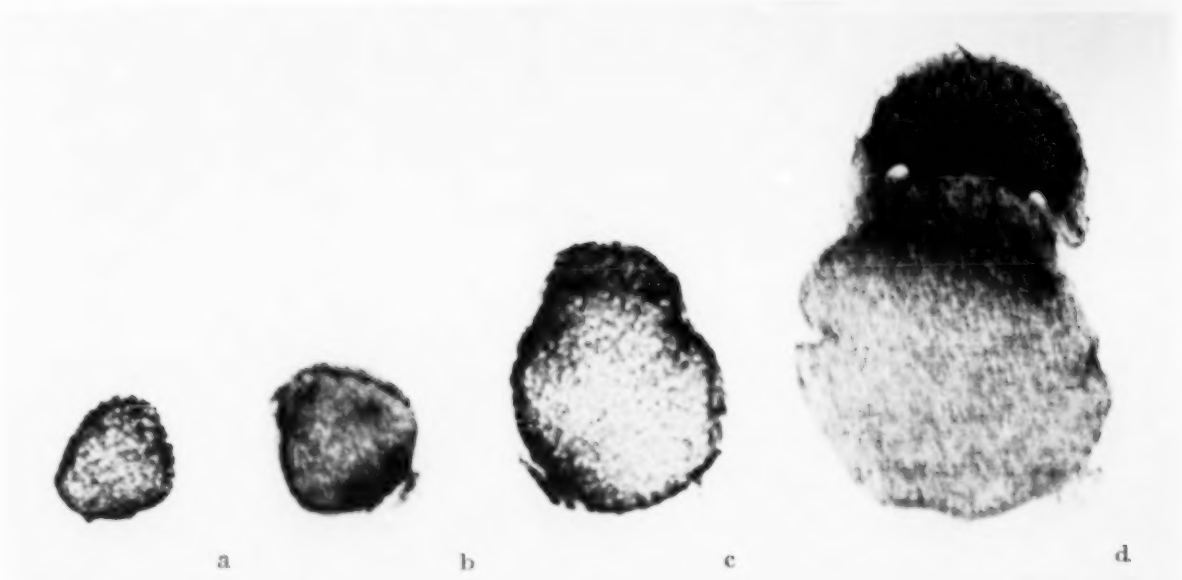

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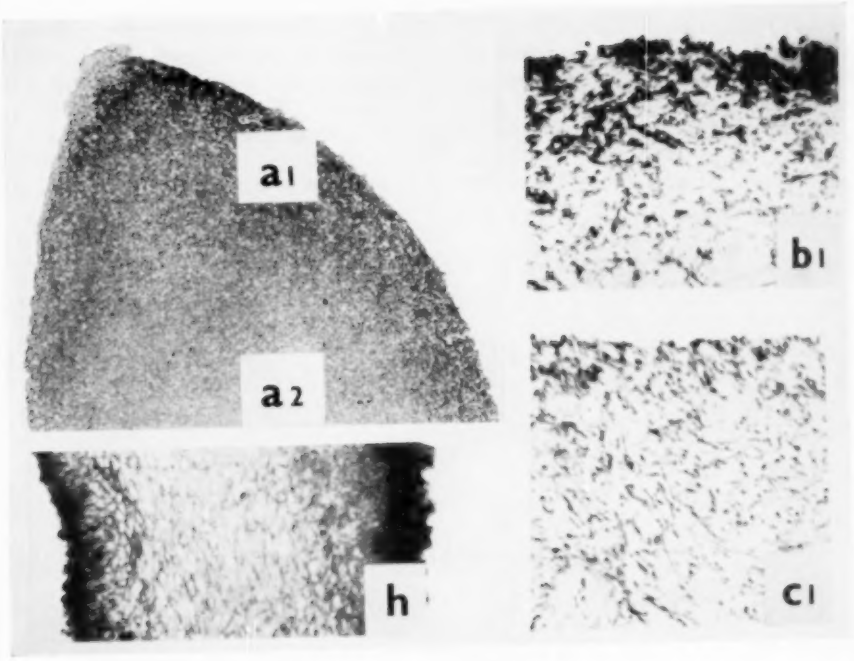

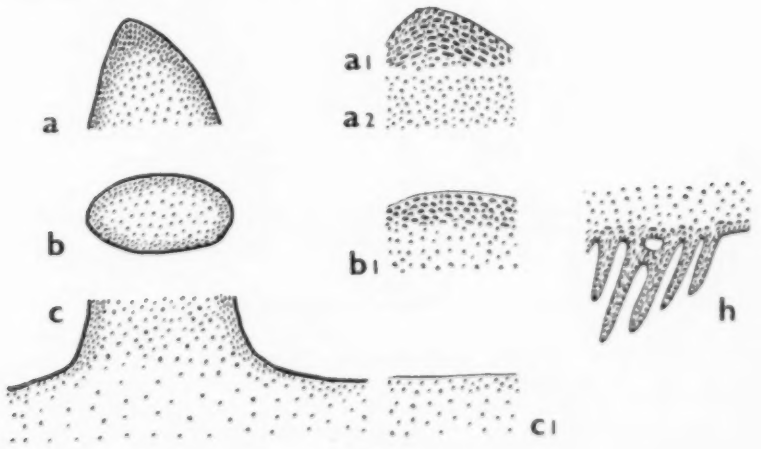

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stage, but were not found after the early button stage. The present studies confirmed Evans' observations. In addition, from squash preparations it was possible to deduce the regions where divisions occurred in the late pinhead and early button stages. Nuclear divisions were found in non-vacuolated cells of the undifferentiated pinhead, and the cap and upper half of the stipe in the differentiating late pinhead stage (Fig. 3c). In the early button stage (1–2 cm. diameter) nuclear divisions were only found in non-vacuolated cells seen in squash preparations of small pieces of tissue removed from the edge of pileus, the developing

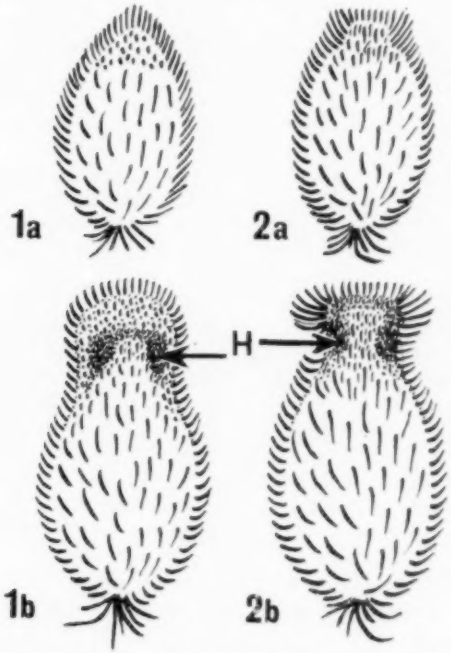

FIG. 5.—Diagram (after Corner, 1935) of longitudinal sections through young fruit bodies showing early stages in two types of Agaric fruit body morphogenesis. 1a, 1b: Hemangiocarpous. 2a, 2b: Gymnocarpous. H: Developing gill tissue.

gill tissue started becoming binucleate, by forming cross walls in the multinucleate cells, by the 1·5–2 cm. diameter stage.

It is interesting to note that the cytological studies are in keeping with the growth measurements and histological studies of Bonner *et al.* (1956). Thus nuclear divisions were found in the regions where increase in the number of filaments and number of cells continued longest. After the 1·5–2 cm. stage mitotic divisions were not found in somatic tissue, a finding which supports the idea that all further growth could be attributed to increase in cell size.

Yet another aspect of morphogenesis contributes to our understanding of the normal pattern of differentiation. During the present studies the growth of the tissues was followed histochemically for the distribution of RNA by studying

stained sections. (Although a squash technique would provide the best way of observing the cytology of entire cells, sections were necessary for observing the orientation of tumour cell types to one another and to normal tissue.) Observation of the distribution of RNA could be expected to indicate the metabolically active cells because cytochemical studies on RNA content show a correlation with the level of protein synthesis in tissues (Caspersson, 1947, 1950; Brachet, 1950; Mirsky, 1951). Also Wade (1955) found that rapid cell division is correlated with a high concentration of RNA rich particles in the cells. Furthermore, *in vitro* biochemical experiments by Gale and Folkes (1954) first provided a direct demonstration that nucleic acids are involved in protein synthesis. (The protein synthesis of fragments of disrupted cells was stopped by the addition of ribonuclease for specific breakdown of RNA. Addition of RNA extracted from the same organism largely restored protein synthesis.) This work has been confirmed by several workers, including Webster and Johnson (1955) and Bridoux and Hanotier (1956). (The latter showed that ribonuclease treatment inhibited incorporation of amino acids into proteins of protoplasts, obtained by dissolving away bacterial cell walls with lysozyme.) These *in vitro* studies are also in agreement with the *in vivo* experiments of Brachet *et al.* (cited by Brachet, 1955, 1958) on living onion root tip cells, where ribonuclease treatment inhibited incorporation of radio-actively labelled amino acids.

In the cultivated mushroom Evans (1955), on the basis of staining reaction with the basic dyes aceto-carmine and pyronin,* concluded that the increased basophilly of the cytoplasm in the Diesel-oil tumour cells compared with normal pinhead cells was due to an increase in RNA synthesis. However, in the present study specific cytochemical staining reactions for RNA showed no increase in the RNA content of the soot-induced T1 cells compared with the N1 cells of the normal pinhead (Fig. 4). (This difference in RNA content between the soot tumour cells and the Diesel-oil tumour cells may be due to a difference in the nature of the tumours, or a difference in technique.) As expected, the soot T2 cells were richer in RNA than N2 cells of the same fruit body, since they were younger cells.

The pattern of the development of cell types to normal maturity and in tumour formation is represented diagramatically in Fig. 6. The N1 and T1 and H cells occupy the deeply staining zones in Fig. 3 and 4, while the N2 and T2 cells are found in the least stained areas.

Since no cytological or histochemical or growth rate differences were found between normal young undifferentiated tissue and the soot induced tumour tissue, it is concluded that the nature of the tumour growth was apparently not abnormal. Their abnormality seems to lie in the continued growth of cells which would normally have ceased doing so, as part of the normal pattern of organisation of the fruit body for its function of spore production.

The studies on tumour production in the cultivated mushroom and the present studies give rise to two views on which cells gave rise to the tumours:

(a) Evans' (1955) view is that *non-vacuolated cells which had ceased dividing were stimulated to divide again.*—This view is based on the fact that tumours did not appear until a stage in differentiation when nuclear division in cells of the pileus had normally ceased (i.e. after early button stage). Yet it was necessary to start treatment of fruit bodies with Diesel-oil at the pinhead stage since it was

* Although not stated, this was actually pyronin B.

not possible to produce tumours unless treatment was started at pinhead stage. Evans found that despite repeated attempts (with direct application and vapour treatment) to induce tumours by commencing treatment after the pinhead stage no tumours were produced. Also in the present experiment repeated attempts to induce tumours by soot treatment after pinhead stage were unsuccessful. It is more likely that the actively dividing cells of the pinhead were affected by the treatment in such a way as to produce imperfect daughter nuclei incapable of

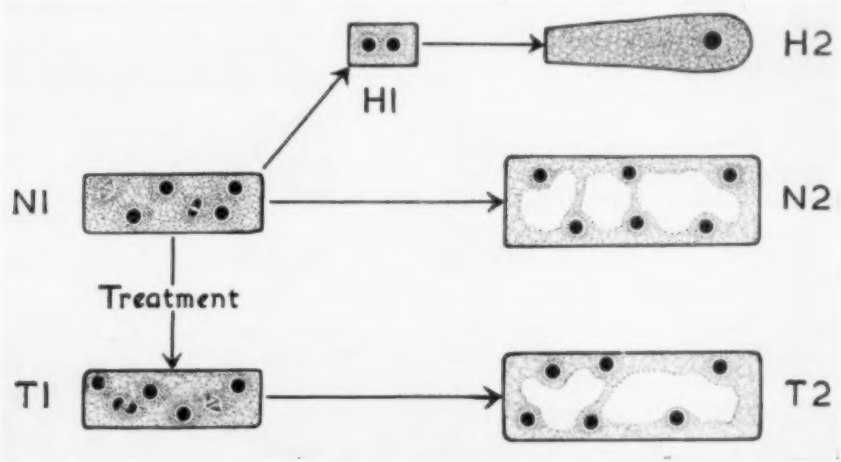

FIG. 6.—Diagrammatic representation of the pattern of differentiation of cell types to normal maturity and neoplasm formation.

Normal cells :— N1 : not vacuolated ; N2 : vacuolated.
H1 : subhymenium ; H2 : hymenium.
Neoplasm cells :—T1 : not vacuolated ; T2 : vacuolated.

co-operating with other cells in the process of fruit body differentiation. This idea gives rise to the second view, which is put forward here :

(b) *The cells which gave rise to the tumours were ones treated at a metabolically active stage with nuclei undergoing mitosis.*—In the case of the Diesel-oil tumours it was necessary to commence vapour treatment at the pinhead stage and give at least seven days' treatment, so that treatment was actually spread out over several stages of development. However, in the case of the soot-induced tumours there can be no doubt as to which stage in development was treated since only the pinheads were treated during their formation and growth through the casing soil. The active carcinogen known to be present in the soot, benzopyrene, is insoluble in water and non volatile—so treatment of the fruit must have been by direct contact in the casing soil. The evidence for the second view is as follows :

(i) Treatment had to be administered at the pinhead stage when mitotic activity was still going on, or no tumours were produced.

(ii) *Accessibility of susceptible cells.*—The fact that tumours were only formed on the upper surface of the pileus and none on the stipe is interpreted as due to

the difference in accessibility of the metabolically active tissues to the carcinogen : —

The absence of tumours of the gill region and the stipe may be attributed to their relative inaccessibility to the carcinogen because of the endogenous development of the pileus. The growth region of the stipe at the stage when mitosis occurs is covered on its outside by tissue which will give rise to the veil (Fig. 3). In animals the fact that most cancers are carcinomas (from surface tissues like epithelium) and that sarcomas (from deep-seated tissues) are least frequent is interpreted as due to the difference in accessibility of the tissues to carcinogenic agents (Cowdry, 1955).

(iii) *It is well known that intermitotic cells are most susceptible to carcinogens* (Cowdry, 1955).—In the mushroom there are two types of cells during differentiation : intermitotics and post-mitotics.

Intermitotics are those cells whose life span depends on the times between division. Their function is to produce new tissue, and reproductive cells. Post-mitotic cells are ones that have finished dividing, and function as units in differentiated tissue until their death.

The intermitotics are of two kinds :

(a) *vegetative intermitotics* (undifferentiated cells) which reproduce their own type and give rise to

(b) *differentiating intermitotics*, so called because, although their nuclei still go through divisions, the cells have developed different specialized properties that distinguish them from undifferentiated cells. The hymenial tissue is an example of this type (also the pileus rim and upper stipe at late pinhead early-button stage). The post-mitotics are cells that no longer undergo mitotic activity. They are the differentiated cells of mature tissues such as stipe and pileus. Normally they are fixed in the post mitotic state, and like the fixed post-mitotics of animals (such as nerve cells) do not revert to mitotic activity, in the differentiated body. Although the mushroom fixed post-mitotic cells and animal post-mitotic cells are capable of mitotic activity in tissue culture, this fact does not affect the present argument because in their natural environment in the differentiated body they are fixed in the undividing condition. (Differentiation in the mushroom is cytoplasmic, since tissue cultures can reproduce the whole organism without variation (Kligman, 1943).)

It appears that in the mushroom the only cells potentially capable of tumour production are the vegetative intermitotics and the differentiating intermitotics, which are the undifferentiated mitotically active cells of the pinhead, the differentiating intermitotics of the pileus rim and top part of the stipe (at the late pinhead and early button stages) and the hymenial tissue. All these tissues except the stipe produced abnormal differentiation in response to carcinogen treatment by Evans (1955). The absence of response in the stipe may have been due to the inaccessibility of the actively mitotic region at its most susceptible stage (late pinhead–early button stage) due to the folding of the developing pileus around the top of the developing stipe. In the Diesel-oil experiment it seems that tumours only arose on fruit bodies where treatment was begun when the mitotically active cells were near the surface of the fruit body. Diesel-oil vapour would not be expected to penetrate deeply because oil and water of the mushroom cells do not mix ! and there is no circulatory system like the blood stream of animals. The position of the soot tumours (Fig. 1) suggests that they were

probably due to a small number of intermitotic cells being affected by treatment at the pinhead stage, probably at the rim of the developing pileus (Fig. 3c). The absence of tumours of the hymenium after Diesel-oil treatment can be attributed to the endogenous mode of pileus development so that gill tissue is not exposed until the veil is burst, at a late stage in pileus development. (Although some vapour may penetrate the veil after the early button stage and be responsible for reticulate gill formation.)

(iv) *Latency.*—One of the characteristics of carcinogenic activity in animals is that there is usually a latent period between treatment and the appearance of tumours (Cowdry, 1955).

Latency between the time of treatment and the appearance of the tumours is one of the features noted for the " soot tumours ". Treatment occurred at the pinhead stage, and tumours did not become apparent until early maturity (just after the veil had ruptured), i.e. the tumours were latent during the button stage. (This does not necessarily mean that the cells which gave rise to the tumours were not active during this time. It is probable that the cells responsible were multiplying, possibly from a very small number of cells affected by the carcinogen.)

In the case of the tumours induced by Diesel-oil vapour by Evans (1955), it was stated that at least seven days' treatment was necessary for tumour production, and since treatment was continuous until the tumours appeared it is not possible to distinguish a period of latency between the time of treatment and the appearance of the tumours. Yet such latency may have occurred, since it was not possible to discriminate which cells at what stage of treatment eventually gave rise to the tumours.

In conclusion it is suggested that the most plausible explanation is that the intermitotic cells were affected by carcinogenic treatment, in such a way that imperfect cells were produced lacking in factors, nuclear or cytoplasmic, responsible for sensitivity of cells to cytoplasmic gradients (Mather, 1948) prevailing during normal differentiation, so that they failed to co-operate with other cells in their united efforts towards reproduction of the organism.

Furthermore the delay in appearance of tumours until the button or mature stage, although treatment was necessary at the pinhead stage, is attributed to latency of the affected cells until their activity became morphologically apparent at a later stage.

These ideas would be compatible with the theory of differentiation of cell types put forward by Rusch (1954) that " The final properties of differentiated cells depend on the particular system of reactions nurtured in the first few cellular generations ", i.e. before they become differentiated cell types. Introduction of new factors such as carcinogens into the environment may influence cell development in such a way that some cells lose their capacity to differentiate from the " vegetatively intermitotic " condition and by their active mitoses become tumour-producing cells.

SUMMARY

1. Diesel-oil soot treatment induced tumours of the pileus.

2. A comparison of the N2 and T2 cells of the same pileus revealed the same number of nuclei.

3. A comparison of the RNA distribution during normal morphogenesis and in the tumour tissue, showed a similarity between the distribution in the tumour tissue and the undifferentiated pinhead.

4. No difference was found between the growth of normal pileus tissue and tumour tissue from the same fruit body in tissue culture.

5. The question about which cells gave rise to the tumours is discussed. The evidence suggests that they were metabolically active intermitotic cells.

The above work was conducted at the Department of Agricultural Botany, University College of Wales, Aberystwyth, under the direction of Professor P. T. Thomas, whose help is gratefully acknowledged. Thanks are also gratefully extended to Professor Alexander Haddow for taking an interest in the use of the cultivated mushroom as an experimental organism, and to the British Empire Cancer Campaign for providing financial support for this work. I am grateful to Mr. K. G. Moreman and staff, particularly Mr. M. J. Docherty, for making photographic enlargements in the Photography Department of the Chester Beatty Research Institute.

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OBSERVATIONS ON MURINE MONOCYTIC LEUKAEMIA INDUCED BY A VIRUS ISOLATED FROM S37 SARCOMA

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THREE different forms of mouse leukaemia resulting from cell-free preparations of S37 sarcoma have been described. Myeloid leukaemia with a large proportion of chloroleukaemia was isolated from this source among others by Graffi (1957). Franks *et al.* (1957, 1959) reported a chronic leukosis associated with overgrowth of large anaplastic mononuclear cells appearing in spleen, liver, kidneys and lymph glands. More recently Moloney (1960) described a lymphoid leukaemia which he also isolated from S37 sarcoma tissue by filtration and differential centrifugation.

The disease described by Franks and his associates arose originally in a Swiss mouse which had undergone S37 sarcoma regression following X-ray treatment. Later it was found that cell-free extracts of diseased spleen or of S37 tumour tissue itself gave rise to the same condition. The present paper describes further observations made on the disease in our own laboratory; a preliminary report has already been presented elsewhere (Bather *et al.*, 1960).

MATERIALS AND METHODS

Mice

Young adult Swiss mice obtained from the Connaught Laboratories, Toronto, Canada, or bred from these animals, were used for all experiments reported here. Newborn animals have frequently been inoculated and develop the disease with the same characteristics as adult mice.

S37 Monocytic leukaemia virus

Two infected Swiss mice were obtained from Dr. W. R. Franks late in 1958. These animals had been inoculated intraperitoneally with a homogenate of infected spleen approximately 2 months previously, and both had palpably enlarged spleens.

Methods of passage

We have employed the intraperitoneal route routinely for virus or cell homogenate inoculations, although infection also occurred when the subcutaneous or intravenous routes were used. Virus preparations were made in two ways, either by centrifugation of spleen homogenates or by centrifugation combined with filtration through a Seitz filter pad. For most experiments filtration was omitted because of considerable lowering of activity, due to adsorption of virus

on the filter. Usually, a 10 per cent spleen homogenate was subjected to 2 cycles of centrifugation of approximately 1000 g for 20 minutes in a swing-out rotor. After each cycle, the supernatant was removed carefully so as not to disturb the centrifuged pellet of cells and debris. Microscopic examination of supernatants never revealed an intact cell. On one occasion a partially purified virus preparation was made by the method employed for Rous sarcoma virus (Bather, 1953). This procedure entails differential centrifugation and trypsin treatment. The final cell-free pellet was highly active.

Histological examinations

Tissues were fixed in 10 per cent formalin or Carnoy's fluid and stained with haematoxylin and eosin for microscopic examination.

Haematological observations

Haematocrits were determined on blood obtained from the end of the tail using capillary tubes centrifuged in a micro-haematocrit head for the International clinical centrifuge (Model CL). White cell counts were made using standard white cell count pipettes and counting chambers. Differential white cell counts were made on smears of peripheral blood stained by Wright's method. A total of 200 cells were examined for each differential count.

EXPERIMENTAL

Fig. 3 shows a very advanced case of the disease in which spleen, liver, kidney, thymus and lymph nodes were all greatly enlarged. This picture usually occurred in mice which lived beyond the main mortality peak and survived 5 months or more. Most animals developed only enlarged spleens before death and, in a few cases, enlarged thymus. A more detailed description of the pathological changes is given in a later section.

Mortality rate and spleen weights

The pattern of deaths following intraperitoneal virus inoculation was essentially similar in all infected groups of animals. Fig. 1 shows the mortality distribution of a group of 66 young adult (5–6 weeks old) Swiss mice having an equal number of male and female animals (upper chart). The lower chart in Fig. 1 gives the range of spleen wet weights of another group of 50 mice of the same age which were sacrificed at intervals after inoculation. This chart also includes the spleen weight range of a saline injected control group. Control spleen weights in these animals ranged from 0·25 g. to 0·68 g.

Gross splenic enlargement was detectable within 10 days after virus inoculation and reached its peak at a time corresponding to the maximum death rate. Splenic rupture, which was reported as occurring frequently in Friend's leukaemia (Metcalf, Furth and Buffett, 1959), was seldom seen in this disease. The largest spleen encountered in this group reached a weight of 4·75 g.

The mortality curve shows only one peak (at 30–50 days) again differing from Friend's leukaemia which shows two distinct peaks, one at 10–30 days (associated with splenic rupture) and one at 50–80 days (Metcalf *et al.*, 1959). Over 90 per cent (62 of 66 animals) in this group died within 3 months of inoculation.

Pathology

The peripheral blood picture was followed at intervals after inoculation of a group of 25 animals with the leukaemia virus. Haematocrit levels and white cell counts were made at the times indicated on the curves in Fig. 2. Each point

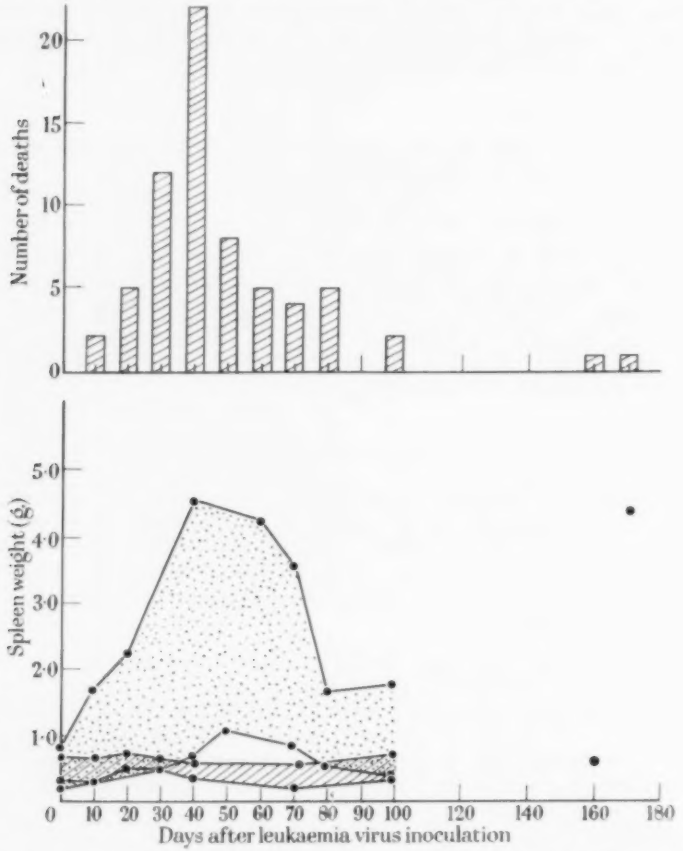

FIG. 1.—Chart showing mortality (upper portion) and range of spleen wet weights (lower portion)* of Swiss mice inoculated with S37 monocytic leukaemia virus. The numbers of mice represented are 66 and 50 respectively.

* Stippled area—leukaemic mice.
Hatched area—controls.

represents the average of measurements made on 3 different animals. The horizontal dotted lines indicate the range of white cell counts found in a control group of 25 mice injected with saline.

The normal haematocrit level of these animals was 40 to 45 per cent and the inoculated mice showed slightly lowered haematocrits by approximately 20 days

after infection. After 2 months, levels were down to 25–30 per cent in many cases.

The total peripheral white count remained steady or slightly low during the first 30 days and then greatly elevated levels were encountered as the disease progressed. In later appearing cases, differential counts showed a marked monocytosis with some erythroblastosis and an increase in lymphocytes. The proportion of erythroblasts appeared to rise steadily, constituting approximately

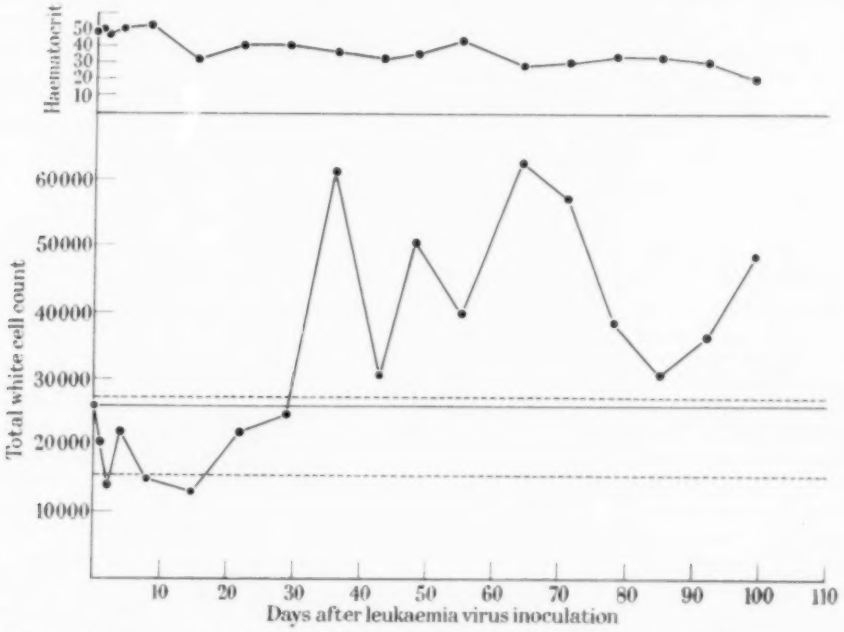

FIG. 2.—Haematocrit levels and total white cell counts of peripheral blood from 25 837 monocytic leukaemia injected Swiss mice. Horizontal lines indicate the average normal white cell counts, with the range, in control animals. Each point is the average of values obtained from 3 different animals taken in rotation during the course of the experiment.

6 per cent at 67 days and 24 per cent at 99 days. In the same period the proportion of lymphocytes dropped from 40 to 20 per cent. Polymorphonuclear cells accounted for some 20–30 per cent of the total count while monocytes made up 13–14 per cent and monoblasts 9–12 per cent. The latter resembled the "Friend" cell described as occurring in the blood of mice in the advanced stages of the virus-induced reticulum cell neoplasm of Friend (1957). Fig. 4 shows the typical appearance of these cells which are characterized by a hyperchromatic nucleus, a denser chromatic arrangement than normal and an increased basophilia in the cytoplasm. The nucleus sometimes showed marked variation in shape and size, in contrast to normal monocytes. Cytoplasmic budding, described by Metcalf *et al.* (1959) as occurring in Friend cells, was often noted and resulted in cytoplasmic masses roughly spherical in shape appearing in the blood smears.

Grossly the enlarged spleens were soft with rounded edges and showed haemorrhagic areas and white foci of neoplastic cells. Microscopically lymphocytic elements had decreased and cells of the erythroblastic series increased. Infiltration of the characteristic large monoblastic cells, already described as occurring in the peripheral blood, was widespread (Fig. 5). These cells are assumed to arise from a neoplastic change in the reticular tissue.

Although the liver showed little or no enlargement during the main period of mortality, it frequently did in the later appearing cases. The organ became greyish in colour, friable, with rounded edges and a granular surface. Microscopically there was marked infiltration of the liver cords by round cells which were not at all localized as in extramedullary haematopoiesis (Fig. 6 and 7). These cells were chiefly composed of erythroblasts, lymphocytes and the monoblast type cells already described. Similar infiltration of the thymus, lymph nodes and kidney also occurred in advanced cases.

DISCUSSION

The disease described in this paper resembles in many respects that described by Friend (1957), especially in the type of abnormal cell predominating in the blood picture in the later stages of the disease. The differences we have noted (e.g. a single instead of double mortality peak and the absence of splenic rupture) are probably not important in differentiating between the two conditions but may simply reflect small differences in response by our Swiss mice which are heterozygous. Friend's leukaemia arose as the result of inoculating a cell-free preparation of Ehrlich ascites cells, and one of the tissues from which the chloroleukaemia of Graffi (1957) was isolated was the Ehrlich carcinoma. Moloney's (1960) lymphoid leukaemia and Franks *et al.* (1957, 1959) monocytic leukaemia both derived from S37. In view of these facts it seems likely that all these virus-induced leukaemias need not be directly related to the tumours from which they were isolated. We agree, therefore, with Moloney (1960) that the virus of S37 monocytic leukaemia like that of his lymphoid leukaemia probably resides in the S37 tumour cells as a " passenger " which happens to favour this milieu. Whether the virus isolated from the leukaemia described here is related to that of Friend (1957) will have to be determined by immunological studies.

We are inclined to regard the characteristic cell found in the peripheral blood and infiltrating the spleen and liver of infected animals as a monoblast and refer to the disease as S37 monocytic leukaemia. As in the case of Friend's disease,

EXPLANATION OF PLATES

FIG. 3.—Mouse dead of S37 monocytic leukaemia approximately 5 months after inoculation. White areas of neoplastic cells in spleen. Spleen, liver, kidney, thymus and lymph nodes all greatly enlarged.

FIG. 4.—Monoblast cells typical of those found in peripheral blood of virus inoculated mice. The upper monoblast seems to be forming a cytoplasmic " bud " and a free " bud " appears beside the lower monoblast. (Wright's Stain—× 1100.)

FIG. 5.—Section through one of the infiltrated areas of an infected spleen showing many monoblasts among the normal erythrogenic cells. (H. & E. × 210.)

FIG. 6.—Section through infected liver in the region of a blood vessel. Monoblasts are evident in the blood and infiltrating the liver sinusoids. (H. & E. × 170.)

FIG. 7.—Neoplastic infiltration of the liver cords of a mouse infected with S37 monocytic leukaemia. (H. & E. × 170.)

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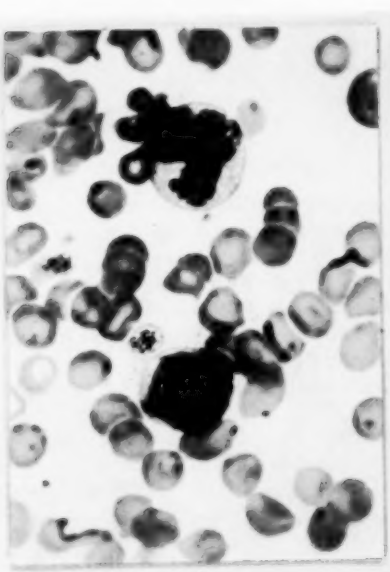

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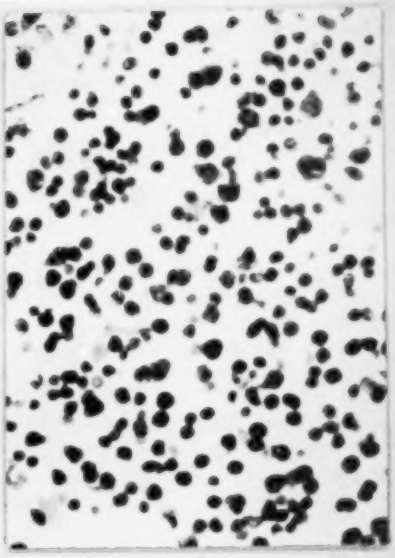

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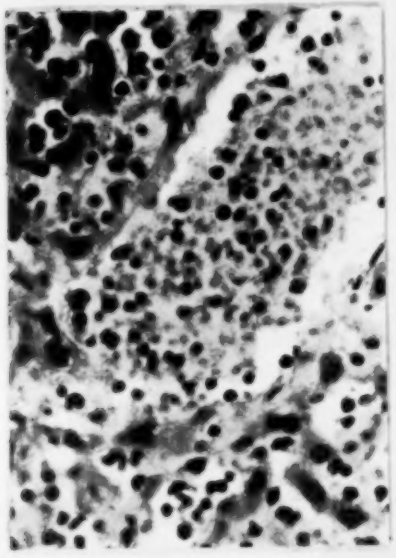

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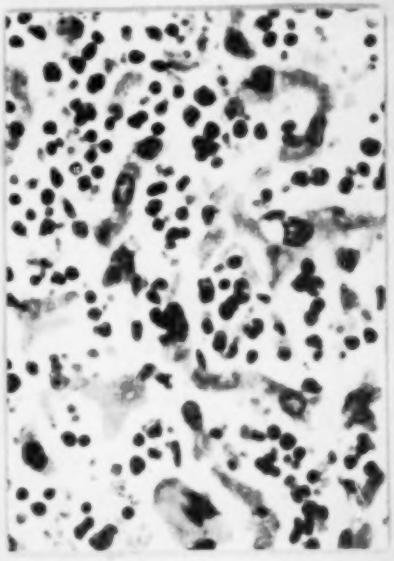

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the erythroblastosis encountered here is probably a secondary effect of the virus, the major cause of death deing due to infiltration of, and interference with, the vital organs by the monoblastic cell. Cytogenetic studies made on these cells are reported by Wakonig-Vaartaja (1960) in a concurrent paper.

SUMMARY AND CONCLUSIONS

A monocytic leukaemia is described which has resulted from infection by a virus originally isolated from S37 sarcoma tissue and from the spleens of animals surviving S37 sarcoma growth and regression.

Advanced cases of the disease are characterized by greatly enlarged spleen, liver and, in some cases, lymph nodes, thymus and kidney. Splenic enlargement is consistent, and is detectable within 10 days after virus inoculation. Maximum mortality rate occurs between 30 and 50 days after inoculation, and is associated with the extent of splenic enlargement and infiltration with erythroblastic and monoblastic cells.

Erythroblastosis and monoblastosis occur in the peripheral blood late in the disease. The monoblasts resemble the " Friend " cell of Friend's reticulum cell sarcoma and exhibit variation in the shape and size of the nucleus, characteristics of staining and cytoplasmic " budding " described for the Friend cell.

All expenses in connection with this work were borne by the National Cancer Institute of Canada. I should like to thank Dr. R. W. Begg for discussions concerning the pathological observations and Miss Irene Sebastian for her excellent technical assistance.

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CHROMOSOMES IN LEUKAEMIAS INDUCED BY S37 AND FRIEND VIRUSES

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CELLS in many neoplasms have a chromosome number higher (aneuploid) than the normal one (Ford and Hamerton, 1956; Levan, 1959; Makino, Ishihara and Tonomura, 1959; Wakonig, 1960*a*, 1960*b*), and it has been suggested that the appearance of aneuploidy is the initial change in neoplasia. Another possibility is that aneuploidy is a secondary change. This appears likely in many types of neoplasms because of the increasing reports of predominantly diploid neoplasms (Bayreuther, 1960; Wakonig, 1960*a*, 1960*b*). In this paper chromosome analyses are reported on 22 cases of the S37 monocytic leukaemia and on 3 cases of Friend's leukaemia. The pathology of the S37 leukaemia is described in an accompanying paper (Bather, 1960).

METHODS

Chromosome analyses were performed mainly on spleen cells of the leukaemic mice, the neoplastic tissue being prepared according to Ford and Hamerton (1956). Colchicine was injected intraperitoneally two hours before the mice were killed. Small pieces of tissue were chopped and pretreated in a hypotonic sodium citrate solution for 25 minutes at 37° C. and then stained with acetic orcein. The chromosome number and any obvious departure from normal morphology were recorded in intact metaphase plates with well spread and well contracted chromosomes.

RESULTS

Table I shows the chromosome number in spleen cells from the monocytic leukaemias induced by the S37 virus. The great majority of the spleen cells were almost or completely diploid, each cell having 40 chromosomes which is the normal number for the species.

TABLE I.—*Chromosome Number in Spleen Cells from Leukaemias Induced by S37 Monocytic Leukaemia Virus*

Group	Number of mice	Number of cells analysed	Percentage of cells with different chromosome numbers		
			40	41	42
A	9	301	100	0	0
B	6	176	96	4	0
C	4	139	91	6	3
D*	3	103	44	49	

* The difference between group D and the other groups was significant at the 1 per cent level (χ^2 test).

In three out of 22 mice more than half of the cells were aneuploid usually with 41 chromosomes. Such a cell is shown in Fig. 2. In most metaphases the extra chromosome could not be distinguished from the others. No gross structural changes were detected in the chromosomes. The chromosomes of the mouse are very similar (Fig. 1) and therefore small structural changes might have been present but would have escaped detection. In sections of tissue not treated with colchicine no aberrant anaphases were noticed.

In repeated passages with cell free extracts, no consistent differences were found in chromosomes between the first and subsequent generations. The leukaemic spleen of one animal was highly (66 per cent) aneuploid in spite of the fact that the donor was highly (96 per cent) diploid.

TABLE II.—*Chromosome Number in Spleen Cells of 3 Mice with Leukaemia Induced by Friend Virus*

Mouse Number	Number of cells analysed	Percentage of cells with different chromosome numbers		
		40	41	42
1	50	100	0	0
2	50	94	6	0
3	50	52	42	6

Chromosome analyses for leukaemias induced with the Friend virus revealed two predominantly diploid cases (Table II), which is in accordance with the findings of Bayreuther (1960). However there was also one highly (47 per cent) aneuploid case in the data of Table II. The cells of this leukaemic spleen had a high incidence (48 per cent) of an easily distinguishable minute chromosome. Studies were made on several more cases of Friend's leukaemia but with a smaller number of metaphases analysed for each ; results were similar to Table II, the majority of cells being diploid.

In leukaemic spleens of all mice injected either by Friend or S37 leukaemia agent, there was a low incidence of tetraploid metaphases. These were not included in the tables.

DISCUSSION

The hypothesis that aneuploidy would be the cause of neoplasia does not hold for the two types of leukaemia studied here ; otherwise most of the spleen cells should have been aneuploid. The occurrence of the few aneuploid cases are rather explained as a secondary phenomenon in the neoplastic cell population, which is partly independent of the controlling mechanism of the body.

In earlier studies a number of diploid cases were found in virus leukaemias of AKR mice (Wakonig and Stich, 1960 ; Wakonig, 1960*a*, 1960*b*). Most of the virus induced mammary tumours of C3H mice studied by Tjio and Östergren (1958) also were predominantly diploid. Furthermore Bayreuther (1960) has summarized his data of various diploid neoplasms induced by viruses. It appears that the virus tumours are more often predominantly diploid than other tumours. This could be explained (Wakonig, 1960*b*) because in such neoplasms the initial change takes place in several cells simultaneously (Duryee, 1956 ; Rubin and Temin, 1958). This would mean rapid development and early appearance of the

neoplasm. In non-viral spontaneous neoplasms the neoplastic change probably takes place in a single cell and many cell generations would be required for the neoplasm to attain observable size. This had been found even in transplanted neoplasms which were started with small inocula (Fisher and Fisher, 1959). The long development would give more chances for secondary evolution and hence aneuploidy.

The suggestion that the aneuploid cases in Tables I and II represent secondary evolution is supported by the lack of specificity : most of the aneuploid cases in groups B, C and D appeared different from each other. This appears to be a result of random changes. Obviously in neoplasms such changes are common as contrasted to the rarity of these in healthy tissue (Wakonig, 1960*b*). Normally the controlling mechanism selects against the progeny of mutated cells, while in neoplastic cells they are easily perpetuated. Similarly in tissue culture where the controlling mechanism is lacking cellular evolution is common.

SUMMARY

Chromosome analyses were made on leukaemias induced by S37 virus and Friend virus. Metaphase plates in the diseased spleens of S37 leukaemias showed predominantly diploid number (40 chromosomes) with only 3 of 22 mice exhibiting an aneuploid value. Two cases of Friend's leukaemia were predominantly diploid, and one aneuploid, with an easily distinguishable minute chromosome. The occurrence of the diploid cases shows that aneuploidy is not the primary cause of neoplasia in these leukaemias. The aneuploid cases are considered as secondary evolution.

All expenses in connection with this work were borne by the National Cancer Institute of Canada.

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EXPLANATION OF PLATES

Fig. 1.—Metaphase with 40 chromosomes from normal spleen. ×4800.
Fig. 2.—Metaphase with 41 chromosomes from leukaemic spleen. ×4800.

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STUDIES ON THE CARCINOGENIC ACTION OF MOTOR ENGINE OIL ADDITIVES

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A NUMBER of preparations are now added to motor oils to improve the performance of automobile engines. In addition to these oil additives, numerous substances are incorporated into modern lubricating oils for a variety of purposes including improvement of oil detergency and prevention of engine wear. Industrial workers, particularly those in the automobile industry are likely to be exposed to these substances by skin contamination and therefore it was considered important to determine whether any of them were carcinogenic.

Initially, studies have been limited to the investigation of various oil additive formulations which are available commercially, particularly for use in automobile engines and the present report describes the results obtained with one preparation.

MATERIALS AND METHODS

Oil additive.—The oil additive chosen for the present study was obtained commercially in sealed cans. The additive, which consists of a soluble formulation in a mineral oil carrier, was transferred to glass-stoppered bottles and stored at room temperature. On standing for several weeks, a white sediment, as yet unidentified, was observed. In the present study, no attempt was made to separate the insoluble material from the clear oil.

Mice.—Stock albino mice (40) equally divided with respect to sex and 6–8 weeks old at the beginning of the experiment. The mice were obtained commercially and maintained on a cubed diet (M.R.C. diet 41) supplemented with fresh greenstuffs plus water *ad libitum*.

Experimental Procedure.—Dorsal hair was removed from mice at the beginning of the experiment and subsequently when necessary. The oil additive (0·3 ml.) was applied dropwise to the skin from an all-glass tuberculin syringe and spread evenly over the clipped area with a glass rod. Mice were treated twice weekly for 45 weeks and then after a rest period of 5 weeks, skin painting was resumed at weekly intervals until the experiment was terminated (456 days).

The mice were examined for the presence of tumours at weekly intervals and animals were sacrificed when it was considered that tumours were malignant, or when they became ill. The position of all tumours was then recorded and specimens of skin, skin tumours and other organs showing gross pathological changes were taken for histological examination.

RESULTS

The following gross changes were observed in mice treated with the oil additive and will be considered separately—(1) Ulceration, (2) Skin tumour formation, (3) Other lesions.

(1) *Ulceration*

The oil additive proved highly irritant to mouse skin and caused marked inflammatory changes which often led to ulceration and secondary infection (Table I). Many of the mice became thin and appeared underweight and it was necessary to suspend treatment with the additive after the 45th week of the experiment. After a rest period of 5 weeks mice were again treated at weekly intervals with the additive and under these conditions there was no recurrence of severe ulceration. The ulcers induced by the additive showed considerable variation in size and shape, some being shallow and well demarcated whilst others were deep and had ill-defined edges.

TABLE I.—*Incidence of Skin Lesions in Mice Treated with Oil Additive*

Type of lesion	Number of mice	Percentage of mice at risk*
Ulcers (simple)	4	—
Ulcers + tumours (benign)	1	17
Tumours (benign)	5	
Ulcers + tumours (malignant)	8	51
Tumours (malignant)	16	
Total tumours	24	69

* Number of mice at risk—35.

Microscopically these lesions could be divided into simple inflammatory ulcers with a floor of granulation tissue and those in which ulceration had become complicated by the development of squamous cell carcinomata (Fig. 1).

(2) *Skin tumours*

Skin tumours were first observed after 135 days and at this time 35 out of 40 mice were still alive (at risk). By the completion of the experiment (456 days) skin tumours had developed in 24 (69 per cent) of the animals at risk and were multiple in 19 (Table I). The tumours varied in size from a few millimetres to over 2 cm. in diameter.

Histological studies showed that tumours in 6 of the mice (17 per cent) were simple hyperkeratotic papillomata with no evidence of invasion. The tumours in the remaining 18 mice (51 per cent) were squamous cell carcinomata,

EXPLANATION OF PLATES

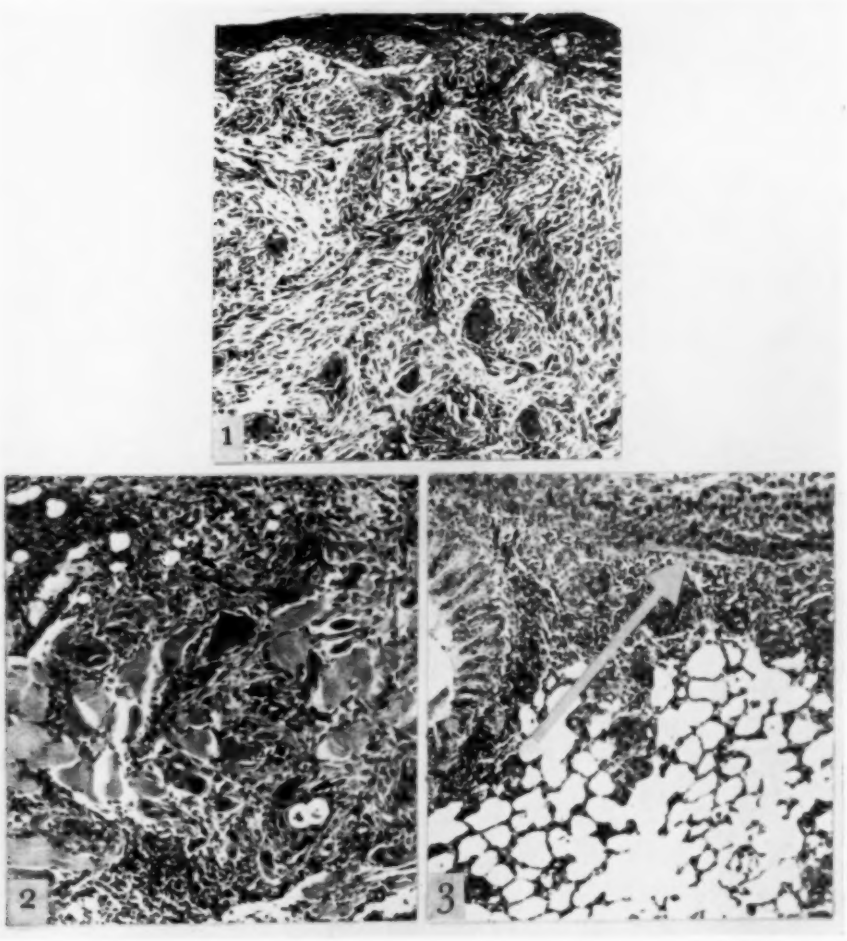
FIG. 1.—Skin ulcer with associated squamous cell carcinoma. H. & E. × 105.

FIG. 2.—Invasion and destruction of muscle by squamous cell carcinoma. H. & E. × 105.

FIG. 3.—Pulmonary metastasis; note invasion of vessel (arrow). H. & E. × 105.

FIG. 4.—Subcutaneous focus of mast cells. H. & E. × 125.

FIG. 5.—Mast cell tumour infiltrating muscle. H. & E. × 125.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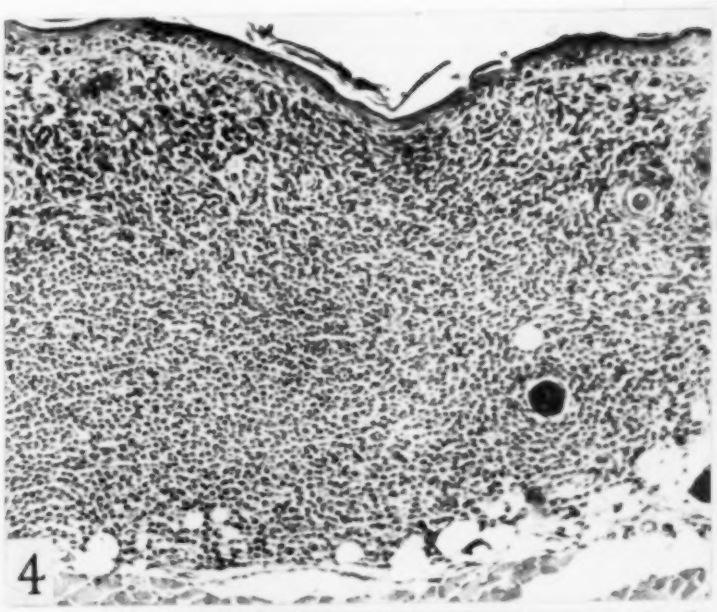

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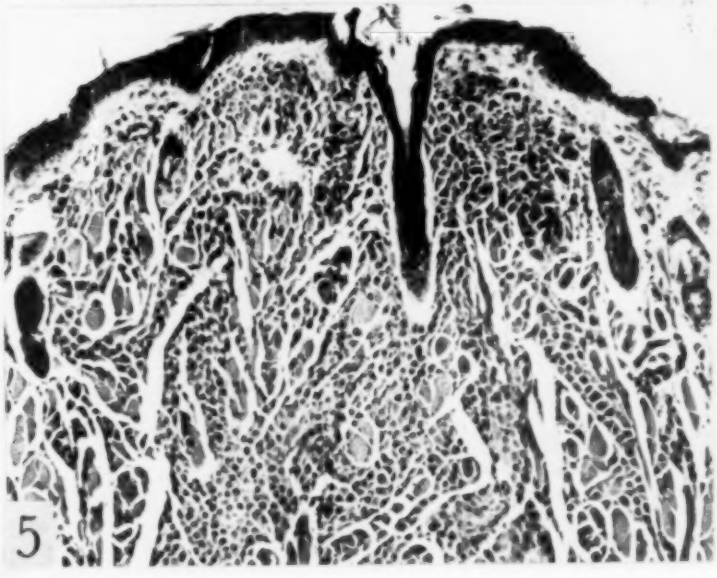

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Baldwin, Cunningham and Pratt.

the cells were usually poorly differentiated, and had invaded the underlying muscle (Fig. 2). In two of these mice secondary deposits of squamous cell carcinoma were also found in the lungs (Fig. 3).

(3) *Other lesions*

Multiple yellowish macules, rarely exceeding 2 mm. in diameter, were noted in 7 mice. In 6 cases these occurred over the treated area of the back, but in one case the macules were observed in the submental region.

Microscopic examination revealed dense aggregates of round or oval cells in the loose connective tissue of the dermis. The cells contained granules which stained metachromatically with toluidine blue at pH 2 thus identifying them as mast cells. Of the 7 cases observed, 6 appeared entirely benign and had excited little cellular reaction (Fig. 4). The remaining case, from the submental region, differed in that it showed invasive characteristics. Many of the cells had infiltrated the underlying muscle and had produced local tissue destruction (Fig. 5).

In one mouse a subcutaneous mass 1·6 cm. in diameter was removed from the perineum. On further examination this was shown to be composed of acini containing small quantities of weakly staining eosinophilic material supported on a fine stroma. The origin of this tumour is obscure.

DISCUSSION

The above experimental findings clearly indicate that the oil additive is carcinogenic for mouse skin and therefore must be investigated as a potential health hazard particularly where there is repeated exposure to the additive.

The components of the additive which may contain carcinogenic substances are the petroleum oil base and a lead naphthenate fraction. This latter component is a crude petroleum product which contains the lead salts of a complex mixture of carboxylic acids, predominantly aliphatic in character, with a cyclopentane ring structure (Thorpe, 1947).

The base oil utilized in the oil additive is a Venezuelan crude oil which has not undergone any thermal reforming. In view of the low carcinogenic response elicited by crude oils of a similar character (Hieger and Woodhouse, 1952) it is unlikely that the carcinogenicity of the additive can be due wholly to carcinogens in the base oil. It is suggested therefore that the other components, particularly lead naphthenate, contain carcinogenic substances or alternatively co-carcinogenic substances which stimulate the response to base oil. In this respect a further component, carbon tetrachloride or 1 : 1 : 1 : trichlorethane, present in small amounts needs to be considered since these halogenated hydrocarbons may possess co-carcinogenic properties.

It is interesting that similar conclusions were reached by Gilman and Vesselinovitch (1955, 1956) in studies with cutting oil formulations which consist of suspensions of sulphurized petroleum oil bases with cutting compound additives. These preparations also produced a much greater carcinogenic response than could be accounted for by base oil alone indicating that the additive agents were possibly carcinogenic.

Clearly further studies are required to elucidate the nature of the carcinogenic agents in this particular oil additive. It is also necessary to determine whether other oil additives both of the soluble type and those containing colloidal suspen-

sions of molybdenum disulphide possess carcinogenic properties, in order to assess the potential health hazard of these preparations.

SUMMARY

1. A commercial oil additive preparation has been examined for carcinogenic activity following skin painting in mice.

2. The additive proved to be irritant for mouse skin and caused inflammatory changes which often led to ulceration.

3. Skin tumours arose in 69 per cent of mice at risk. Histological examination showed that the tumours in 51 per cent of mice were squamous cell carcinomata.

4. It is considered essential that further studies be carried out with a variety of oil additives containing both soluble and insoluble formulations in order to assess whether these preparations represent a potential health hazard.

We are indebted to Miss R. Ellis and Mrs. M. Marshall for technical assistance and to Mr. A. L. E. Barron for the photo-micrographs.

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THE EFFECT OF OESTRONE ON THE INCIDENCE OF MAMMARY CARCINOMA AND ADENOMA IN C_3Hf BREEDERS AFTER LATE OVARIECTOMY

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THE effects on the incidence of spontaneous mammary carcinoma of breeding and of age in C_3Hf mice have been recorded (Pullinger and Iversen, 1960). None of the tests done to detect the mammary tumour agent disclosed its presence in this strain (Pullinger, 1960*a*). A reduction in mammary tumour incidence in former breeding females after ovariectomy alone was reported (Pullinger, 1960*b*). In the present paper the incidence of mammary carcinoma and adenoma in C_3Hf breeders after late ovariectomy with subsequent treatment with oestrone is described.

MATERIALS AND METHODS

Surplus breeders of generations F5 to F16 of the pure line C_3Hf colony were ovariectomised at the age of 9 to 11 months. The operation and subsequent management were the same as that recorded for ovariectomy alone (Pullinger, 1960*b*) except that the animals were treated thereafter with oestrone once weekly for 30 weeks. One experimental group comprising 30 animals received 5 μg. weekly, the other of 23 received 10 μg. weekly. The oestrone was dissolved in acetone (0·01 per cent) and applied to the whole area of the clipped backs in measured doses of 0·05 and 0·1 ml. respectively. The treated animals were allowed to live out their lives and were killed only when they had developed tumours or were about to die. At necropsy the operation sites were examined to ascertain that no ovarian fragments remained. Adrenal glands, tumours and nipple regions from samples of mice from both groups were fixed for histological examination. All 10 nipple regions from samples of mammae were bulk-stained to count adenomas. The samples were selected throughout the experimental period according to the state of preservation of the cadavers and sometimes to time available for microdissection. In specimens where the degree of involution did not allow certainty in count, or where a mammary carcinoma had overgrown most of the nipple region, accurate counts were not possible and were not attempted. Two of the carcinomas were grafted into C_3Hf hosts.

RESULTS

5 μg. oestrone weekly

In this group 2 out of the 30 animals (= 6·6 per cent) developed mammary carcinoma. Both tumours were of type B (Dunn, 1959). One of the tumour-bearing animals died after having received a total dose of 85 μg. of oestrone (out

of 150 μg. possible) and one non-tumour animal died after a total dose of 110 μg. oestrone (Tables I and II).

TABLE I.—*Mammary Carcinoma Incidence after Ovariectomy and Substituted Oestrone in C_3Hf Former Breeders and Numbers of Litters*

	Ovariectomised breeders			
	5 μg. Oestrone		10 μg. Oestrone	
Number of Litters	Number of Females	Number with Carcinoma	Number of Females	Number with Carcinoma
1	0	0	1	0
2	0	0	2	1
3	19	0	4	1
4	8	2	5	2
5	2	0	4	0
6	0	0	1	0
7	0	0	3	1
8	1	0	2	1
9	0	0	1	0
Totals	30	2	23	6
Per cent		6·6		26

TABLE II.—*Mammary Carcinoma Incidence in Former C_3Hf Breeders after Ovariectomy and Substituted Oestrone with Survival Ages*

	Ovariectomised breeders			
	5 μg. Oestrone		10 μg. Oestrone	
Age at death, in months	Number of Females	Number with Carcinoma	Number of Females	Number with Carcinoma
12	0	0	0	0
13	0	0	1	1
14	0	0	0	0
15	1	0	0	0
16	1	1	2	0
17	0	0	0	0
18	0	0	0	0
19	2	0	1	0
20	1	0	2	0
21	0	0	4	2
22	1	0	2	1
23	1	0	1	1
24	3	0	0	0
25	1	0	2	0
26	0	0	1	1
27	5	0	1	0
28	6	0	1	0
29	3	0	3	0
30	1	0	2	0
31	3	0	0	0
32	1	1	0	0
Totals	30	2	23	6
Per Cent		6·6		26

Eighteen mammary glands were examined by bulk-staining and of these 12 bore adenomas and the total number of adenomas was 87 (Table III). The

findings at necropsy were : 6 with hepatoma ; 2 with lung adenoma ; one haemangio-endothelioma ; 5 overt lymphomas ; 3 squamous carcinomas ; 1 with several bladder calculi which did not cause apparent symptoms.

TABLE III.—*Adenomatous Nodule Incidence after Ovariectomy and Substituted Oestrone in C_3Hf Breeders*

	Number of mice examined	Number with Nodules	Percentage with Nodules	Total Nodules	Average Number per Mouse
Ovariectomy only	25	11	44	33	3
Oestrone 5 μg.	18	12	66·6	87	7·2
Oestrone 10 μg.	14	14	100	239	17·0

Eighteen pairs of adrenal glands were examined microscopically. Type A and compact fasciculata cells were present in 11 and B cells in 15, in two of which the cells were hyperplastic. There were no cortical carcinomas. Two medullary carcinomas were found in 2 mice and 2 others had each one accessory adrenal gland. The latter were composed of compact fasciculata cells. Adrenal cell nomenclature is in accordance with that of Woolley and Little (1945). Compact cells of the zona fasciculata in C_3Hf mice were described previously (Pullinger, 1959).

10 μg. oestrone weekly

In this group 6 out of 23 (26 per cent) developed mammary carcinoma. Two tumours were of type A, one of type B, 2 of type C and 1 was an adenoacanthoma. Out of a possible total of 300 μg., one female which developed a mammary carcinoma received 50 μg. Two others without carcinoma had 190 and 200 μg. respectively. Of 14 samples of mammary gland, 14 bore adenomas and the total number of adenomas was 239 (Tables I, II and III). Of 22 females that lived to 16 months or more 3 had hepatoma. One mouse had an osteogenic sarcoma of the tail which was excised but metastasised to the lungs. Four of the 23 were found at necropsy to have urinary calculi. None of the calculi had caused hydro- or pyonephrosis nor death. None of these 4 had mammary carcinoma. No pituitary enlargements were found in either experiment.

Grafts from 2 mammary carcinomas grew in totals of 5 out of 5 females and in 5 out of 6 males.

Survival data and numbers of litters for ovariectomy only and for the 2 experiments here recorded are given in Fig. 1 and 2. Fertility in the group given 5 μg. oestrone weekly was slightly inferior to that given 10 μg. The impression was gained that breeding capacity was lessened or delayed by separation of groups of females after weaning before being mated. Groups were used for 5 μg. but for 10 μg. the females remained from birth with their brothers.

DISCUSSION

Treatment *per cutem* was preferred to multiple subcutaneous injections of oestrone in oil. According to Emmens (1950) either method is valid for assay purposes. In assays of dose-responses in RIIIf mammary glands absorption by this route was found to occur (Iversen, 1957). Application through the skin

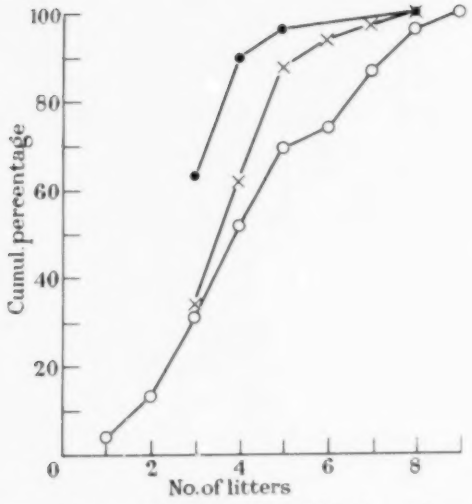

FIG. 1.—Represents the cumulative percentage of breeders and number of litters.
× = Breeders ovariectomised only.
● = Breeders ovariectomised and given 5 μg. oestrone weekly.
○ = Breeders ovariectomised and given 10 μg. oestrone weekly.

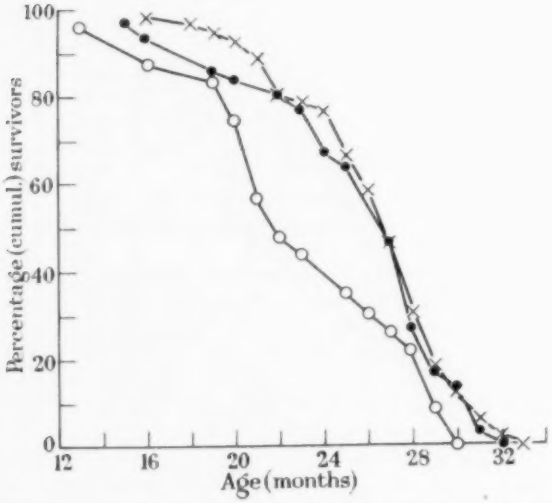

FIG. 2.—Represents the cumulative percentage of survivors and age in months.
× = Breeders ovariectomised only.
● = Breeders ovariectomised and given 5 μg. oestrone weekly.
○ = Breeders ovariectomised and given 10 μg. oestrone weekly.

avoids possible spoiling of nipple regions that are to be used for adenomatous nodule counts.

Previous attempts to test the oestrone dose and tumour response in RIIIf breeders and virgins (Pullinger, 1955, 1957) had been made with measured dosage per mouse and this method was preferred. In the experiments of Boot and Mühlbock (1956) two levels of oestrone of 125 μg. and 2000 μg. per litre were maintained in the drinking water of C_3Hf and C_3He virgin females. Both substrains have the same genetic constitution by derivation as the one used here. Tumour incidence in intact and in mice ovariectomised at 150 to 250 days (Fig. 8) maintained under these dose levels did not differ materially. It was greater at the higher dose level and reached an incidence characteristic for breeders of these strains.

Urinary calculi of ammonium, calcium and magnesium phosphates were found in 4 out of our 23 former breeding females, ovariectomised and given 10 μg. of oestrone weekly but none had caused any deaths. In 108 intact breeders of the reference group (Pullinger and Iversen, 1960) no breeders with urinary calculi had been seen. No higher dose level of oestrone was tested on account of these observations and because experience with post-pubertal females indicated that, for the same reason, 10 μg. weekly is outside physiological limits for this oestrogen. Previously, Schenken, Burns and McCord (1942) reported the incidence of urinary calculi in untreated intact and in intact C_3H males and females treated with α-oestradiol benzoate. In males the incidence of calculi was increased after treatment but not in females. This discrepancy between results in C_3H and C_3Hf females could be due to earlier deaths of the C_3H from mammary carcinoma associated with the mammary tumour agent.

Observations and experiments thus far concluded indicate that the breeding factor increased the number of females with mammary carcinoma from 2 per cent in non-breeders to 25 per cent and adenoma from 29 per cent in non-breeders to 80 per cent. When ovaries were removed after an adequate breeding stimulus, as measured by the number of litters born prior to ovariectomy, carcinoma incidence was reduced to that of non-breeders, or if the one tumour bearer that had an adrenal cortical carcinoma with evidence of oestrogen secretion is excluded, to nil. Females with adenomatous nodules fell from 84 per cent among those in the reference group which had borne roughly comparable numbers of litters to 44 per cent after late ovariectomy. When oestrone near in amount to the limit of tolerance was substituted after ovariectomy, the incidence of spontaneous carcinoma that can be expected for intact breeders was realised. Adenoma incidence reached 100 per cent. These findings show that breeding determined the potentiality for a greater incidence of tumours than in non-breeders but that continued high-level exposure to oestrone in later life was necessary to realise this potentiality. The actual incidence was related quantitatively to the dose level of oestrone. Whether oestrone alone or in combination with other oestrogens is able in virgin females to replace the breeding factor is being tested. The results of Boot and Mühlbock (1956) showed that oestrone alone is effective and our figures to date support such a conclusion. Whether or not oestrone similarly applied after late ovariectomy of non-breeders would be effective in realising as high an incidence as in former breeders requires to be tested by continued high level dosage for the same period of their lives. The answer, if affirmative, might supply a clue to the incidence in human nulliparous females. The incidence in

the latter is generally considered to be even higher than in multiparae. Does oestrogen secretion continue at a high level during the climacteric ? Identity of human and mouse oestrogens is not assumed, merely similarity of action, mediated through the pituitary gland, on mammary epithelium.

The existence or not in this strain of small residual amounts of mammary tumour agent perhaps remains disputable. The recent publication of very high magnifications of 130,000 to 360,000 times of the agent stained *in situ* with lead hydroxide by Bernard (1960) makes possible the identification of individual and possibly unique particles of the mammary tumour agent. The problem of explaining discrepancies between negative biological tests and the presence of morphological images of the mammary tumour agent is discussed by Bernard. Continued breeding and observation of this C_3Hf strain and experience of substituted oestrogen in post-pubertal females and breeders has failed to provide evidence of the presence of the agent. It is considered that mammary tumours of this C_3Hf strain are due to a heritable tendency and that their development and actual incidence depend upon the level of oestrogen of their hormonal environment, especially in later life.

SUMMARY

Two out of 30 C_3Hf breeders ovariectomised at 10 to 11 months of age and subsequently treated with 5 μg. oestrone weekly developed mammary carcinoma ; 12 out of the 18 examined bore adenomatous nodules.

Six out of 23 breeders similarly ovariectomised but treated with 10 μg. oestrone weekly developed mammary carcinoma ; 14 out of 14 bore adenomatous nodules. Continued high level exposure to oestrone after breeding and ovariectomy brought the incidence from 2 per cent, found after ovariectomy only, to 26 per cent which is the spontaneous incidence for breeders of this strain.

I am indebted to Dr. S. Iversen for Fig. 1 and 2.

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FAILURE OF METHYLCHOLANTHRENE TO INDUCE BREAST TUMOURS IN MALE IF MICE WHEN ADMINISTERED BEFORE STIMULATION OF THE BREAST TISSUE BY OVARIAN SECRETION

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In the chemical induction of breast tumours in mice, it is well known that the interaction of ovarian hormones during the period of administration of the carcinogen, and afterwards, plays a large part in determining whether or not tumours will arise.

Much of the work in this field has been done with the IF strain of mice established by Bonser and described by her (Bonser, 1938), using methylcholanthrene (MC) as the carcinogen. Virgin females of this strain do not develop breast tumours spontaneously, but yield a high incidence after MC treatment (Orr, 1943 and 1946; Marchant, 1955). Such tumours do not contain mammary tumour agent (Dmochowski and Orr, 1949). Bonser (1954) found that in the induction of breast tumours after ovariectomy, administration of oestrogen alone during the period of carcinogen treatment was unable to replace ovarian secretion. However, a high incidence of tumours was restored when progesterone was given in addition to oestrogen (Jull, 1954), or when an IF ovary was grafted subcutaneously (Marchant, 1958). The sensitivity of the breast tissue of intact virgin female mice of the IF strain to carcinogenesis by MC may be attributable to the fact that spontaneous pseudo-pregnancy is common when these mice are caged in groups, indicating that high levels of progesterone are acting.

The role of progesterone has also been demonstrated in experiments with male mice. Intact IF males do not develop breast tumours after MC treatment alone (Orr, 1943), but they will give a similar yield to virgin females if treated with oestrogen and progesterone during the period of carcinogen treatment (Jull, 1954). The same dose of oestrogen, or of progesterone alone, was ineffective. However, Orr (1943) obtained a good yield of tumours in males treated with oestrogen and MC throughout life. Jull (1956) has shown that MC mimics the action of progesterone on breast tissue by causing development of acini in the breast of castrated male mice primed with oestrogen. He has suggested that in Orr's experiment the continued application of MC was acting in a similar manner to progesterone in addition to its action as a carcinogen. Castration of male IF mice and subcutaneous implantation of an ovary prior to MC treatment, will also give a high yield of breast tumours (Marchant, 1958).

In all the experiments quoted above, breast tumours were obtained when the ovarian hormones were acting during, and in most cases after, the period of administration of the carcinogen. Orr (1956) describes briefly an experiment in which male IF mice, treated with MC before treatment with high levels of oes-

trogen, failed to get breast tumours. The present experiments are an extension of this idea to separate in time the carcinogenic and hormonal stimuli. They are designed to see whether carcinogen treatment of male animals, which have rudimentary breasts only, would be effective in eliciting tumours if followed by adequate hormonal stimulus for breast development. Since there is some evidence that androgens inhibit breast tumour development, some of the animals had their testes removed before treatment with the carcinogen began.

MATERIALS AND METHODS

Animals.—Adult male mice of the IF strain were used. They were housed in groups of 5 and fed on rat cube, known as the Thompson diet, with water *ad libitum*.

Carcinogen administration.—The carcinogen administration used on all groups of animals consisted of a total of 8 applications at fortnightly intervals of a 0·5 per cent solution of 20-methylcholanthrene (MC) in olive oil. An average dose of 0·50 ml. (= 2·5 mg.) of MC was applied all over the body surface each time.

Hormonal stimulus.—Testes were removed from the animals at various stages in the experiments. The hormonal stimulus for breast growth was provided by the subcutaneous implantation on one flank of the animal of an ovary from an adult IF female.

Necropsy.—Sample animals in each experiment were killed at the end of carcinogen treatment to determine the extent of breast tissue development at that stage. Other animals were examined regularly for the development of tumours and were examined *post mortem*, or were killed when they seemed unlikely to survive further. Whole-mount preparations of breast tissue from some of these animals were prepared by fixation of the pelts bearing the breast tissue in Bouin's fluid. The breasts were subsequently stripped from the skin and stained with Mayer's haemalum.

RESULTS

Experiment 1. *Intact males treated with carcinogen, then castrated and ovary grafted*

Carcinogen was administered to 38 young adult IF male mice. Two weeks after the last painting, their testes were removed and one ovary from an adult IF female was grafted subcutaneously.

Two mice of this group were killed at the end of carcinogen administration, i.e. before ovary grafting, and their breast tissue examined. One showed the normal male picture of a very limited duct system and no acini. The second showed slight development of the ductal tree with profuse, regular acinar clusters.

The remaining 36 mice survived a mean time of 8·4 months (range 5·3 to 14 months) from the first MC treatment. The 2 which died earliest showed lymphomatosis. Skin tumours, usually multiple, arose as papillomas in 19 of the 21 mice which survived more than 7 months. Almost all had developed at least one squamous carcinoma at the time of death.

No breast tumours developed. Whole mounts of breast tissue were prepared from 3 of the mice. Two, dying 9·8 months from the first MC treatment, showed slight development of the ductal tree, but no acini. The other, dying after 14 months, showed similar duct development and good acinar development.

Experiment 2. *Castrated males treated with carcinogen, then ovary grafted*

Twenty-seven young adult male IF mice were castrated. Four weeks later carcinogen administration began. Two weeks after the last painting, an ovary from an adult IF female was grafted subcutaneously.

One mouse was killed at the end of carcinogen administration. No breast tissue could be detected in it.

The remaining 26 mice survived a mean time of 8·4 months (range 6·3 to 10·5 months) from the first carcinogen treatment. The 2 dying earliest had lymphomatosis. Skin tumours developed in 22 of the remainder. Again they were frequently multiple, arising first as papillomas and later developing into squamous carcinomas in most cases.

No breast tumours developed. Breast tissue of 15 animals was examined. All showed development of the ductal tree, in most cases approximating to that of virgin females. In 2 cases there were no acini. In 9, acinar development was sparse and in the remaining 4 examined there were dense acinar clusters. Two of the latter mice were amongst the last survivors of the group, indicating that the degree of development may have been to some extent due to the length of time the ovary had been present.

Experiment 3. *Castrated males treated with carcinogen. No ovarian graft*

Twenty-two young adult male IF mice were castrated. Five weeks later carcinogen administration was begun. No ovarian grafts were given.

One mouse was killed at the end of carcinogen administration. No breast tissue could be found in it.

The remaining 21 mice survived a mean time of 8·8 months (range 4·8 to 10·5 months) from the first MC treatment. Five of the 7 dying first had lymphomatosis. Skin tumours developed in 11 of the remainder, usually as papillomas going on to squamous carcinomas.

No breast tumours developed and no breast tissue could be found in 2 mice which died 8·3 months after carcinogen administration began.

Previous experiment (*Marchant*, 1958)

In the previous experiment, male IF mice were castrated and ovary grafted 2 weeks before carcinogen administration. The mean survival of the animals was 7 months (range 4 to 11 months) from the first MC treatment. Seventeen of the 27 mice (63 per cent) developed breast tumours. The results of all these experiments are summarised in Table I.

TABLE I.—*The Effect of Castration and Ovary Grafting on the Induction of Breast Tumours by Methylcholanthrene (MC) in Male Mice of the IF Strain*

Experiment	Number of mice	Stages in Treatment			Mice with Breast Tumours	Mean Survival (Months)
		1	2	3		
1	36	MC	Castrated & ovary grafted	—	0	8·4
2	26	Castrated	MC	Ovary grafted	0	8·4
3	21	Castrated	MC	—	0	8·8
Marchant (1958)	27	Castrated & ovary grafted	MC	—	17	7·0

DISCUSSION

Normal male IF mice have a very limited duct system with no acini. When such mice are castrated and grafted with an ovary from an IF female, their breast tissue soon begins to grow. The ductal tree of each breast increases in area and becomes more elaborately branched, and eventually acini usually appear (Marchant, unpublished).

In the earlier experiment, which resulted in the appearance of breast tumours (Marchant, 1958), the carcinogen was administered to such mice during the period when the breast tissue was receiving the stimulus to growth. The ovarian graft also remained after MC treatment was completed. In this case there was tissue on which the carcinogen could act and further stimulation of this tissue to grow continued after completion of the carcinogen treatment.

The results of the present experiments show that MC treatment was unable to elicit breast tumours when it was given to normal, or to castrated, male IF mice before hormonal stimulation of breast growth by an ovarian graft. Although it could not be detected, rudimentary breast tissue must have been present in the castrated mice of experiments 2 and 3, to give rise to the marked development which occurred following ovarian implantation in experiment 2. It would appear that rudimentary breasts are insensitive to the carcinogenic action of MC, even when subsequently provided with an adequate growth stimulus in the form of an ovarian graft.

A somewhat puzzling result occurred in experiment 1, where the carcinogen was applied to intact males. In view of the paucity of sensitive tissue on which the carcinogen could act in normal male IF mice, it would seem unlikely that breast tumours would result, even after subsequent stimulation of breast growth. Although they did not develop, the surprising thing was that one of the 2 samples of breast tissue taken at the end of MC treatment showed considerable stimulation of growth, particularly of acini. If a good proportion of the mice had had well-developed breasts at this time, it would seem that the carcinogen had itself stimulated the breast tissue to grow and, in so doing, provided itself with tissue on which to act—at least in the later stages of the experiment. One might, therefore, have expected some breast tumours to develop in this group of animals, particularly after the subsequent stimulation of the breast tissue by the castration and ovary grafting. Further evidence about the state of male IF breast tissue after MC treatment is being sought. In the meantime, the present observation would confirm Jull's (1956) demonstration of the progesterone-mimetic action of MC in stimulating acinar growth.

In the above experiments, it is interesting to note that the breast development brought about after MC treatment alone, or MC treatment followed by ovary grafting, showed none of the focal irregularities of growth which may be seen in fully-developed mouse breasts that have received carcinogen treatment.

SUMMARY

Intact male IF mice were given 8 fortnightly paintings of olive oil, each containing 2·5 mg. of methylcholanthrene. They were subsequently castrated and grafted subcutaneously with an ovary. The breast tissue grew under ovarian stimulation, but failed to become tumorous.

Castrated mice treated with the carcinogen also failed to develop breast tumours, whether subsequently grafted with an ovary, or not.

Skin tumours appeared in the majority of animals.

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DEOXYCYTIDYLATE DEAMINASE AND THYMINE CATABOLIC ACTIVITY OF TRANSPLANTED MOUSE AND RAT HEPATOMAS AND THEIR HISTOLOGY

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PITOT and Potter (1960) and Potter, Pitot, Ono and Morris (1960) very recently studied various normal tissues and rat hepatomas for the presence of deoxycytidylic acid (dCMP) deaminase (compare also : Maley and Maley, 1959). The enzyme could be demonstrated in embryonic rat liver, in adult liver after induction of bile duct cell proliferation and in a number of the rat hepatomas. Since parenchymal cells of normal adult rat liver did not appear to contain the enzyme, it was considered likely that rat hepatomas which showed dCMP deaminase activity were derived from bile duct or closely related cells (Pitot and Potter, 1960). All rat hepatomas studied by Potter *et al.* (1960), except one, were unable to convert [2-^{14}C] thymine to $^{14}CO_2$, but rat liver was markedly active in the latter respect. These findings pointed to the non-uniform metabolic behaviour inherent to individual tumours, a phenomenon frequently encountered in this laboratory among mouse and rat hepatomas and other tumours (Emmelot, Bos, Brombacher and Hampe, 1959).

Experiments directed towards finding a consistent difference between the enzymic profiles of normal and cancer tissues may ultimately help to clarify the cause and the nature of neoplastic growth and provide clues for a rational chemotherapy. The great diversity in results makes it imperative to concentrate on a few tumour types and to study these extensively. Even then, many tumours of a given kind have to be studied in order to obtain a sufficient amount of data for any valid conclusion, in view of the non-uniform enzymic properties of even those tumours which originate from the same cell type, *e.g.* mouse hepatomas (Emmelot *et al.*, 1959 ; Levintow, 1954). Moreover, the question has to be answered first whether or not one has an appropriate normal cell for comparison. This requirement will undoubtedly restrict the choice of the kind of tumours to be investigated. The system mouse-liver-hepatoma appears a suitable one from the point of view of comparability of cell types but a restriction is imposed on the system rat-liver-hepatoma by the fact that the rat hepatoma may be derived from parenchymal and/or bile duct cells.

In the studies of Potter and co-workers a beginning has been made with the investigation of two important problems. First, may the presence of a certain enzyme, such as the dCMP deaminase, in a rat hepatoma serve as a marker for the cell origin of this tumour (Pitot and Potter, 1960 ; Potter *et al.*, 1960) and, secondly, is the deletion of catabolic enzymes of nucleic acid metabolism a consistent feature accompanying neoplastic growth (Potter, 1958, 1960 ; Potter *et al.*, 1960), and if so, is the deletion of qualitative or of quantitative significance ?

In other words, is the deletion of nucleic acid catabolic enzymes a *conditio sine qua non* for cancer or is it promoting cancerous growth? In the latter case some measure of correlation between the particular enzyme profiles and the growth rate of the tumours, or more generally, the stage of tumour progression (Weinhouse, 1960) might exist. Some evidence of such a correlation in regard to thymine catabolism was obtained (Potter *et al.*, 1960).

Since for the elucidation of these highly interesting questions the study of as many hepatomas as possible is called for, Dr. Potter encouraged us to test some of the hepatomas kept in our laboratory for the above enzymes, and kindly provided us with the necessary information before the publication of the papers referred to above. The present paper is concerned with the results of these studies, carried out on three mouse and four rat hepatomas of which other metabolic properties had been (Emmelot *et al.*, 1959) or were being investigated at that time.

MATERIALS AND METHODS

Tumours.—The mouse hepatomas T 28012 and T 26473 were of spontaneous origin and transplanted subcutaneously on (C57 Black × DBA)F_1 and (O_{20} × DBA)F_1 mice, respectively. Mouse hepatoma CBA 244 had been induced by feeding *o*-aminoazotoluene to a female CBA mouse and was subsequently carried by animals of the same strain. The former two tumours had been transplanted during more than 5 years, the latter tumour was studied in its 11th and 12th transplant generation.

The rat hepatomas had been induced by feeding 4-dimethylaminoazobenzene to female rats of the inbred strain R-Amsterdam, maintained on a polished rice diet. The tumours were transferred intraperitoneally in rats of the same strain. Transplant generations tested for enzyme activity were in the case of hepatoma BY 225 : 98, 101 and 102 ; BY 446 : 11, 13, 17, 20, 22 and 26 ; BY 448 : 9, 10, 11 and 20 ; and BY 463 : 3, 4 and 7. The transplants of hepatomas BY 225, BY 446 and BY 448 were harvested 6–8, 9–12, and 10–12 days after inoculation, respectively. The transplants of hepatoma BY 463 were grown from 3 to 5 weeks, depending on the transplant generation. The hepatomas were studied histologically using conventional techniques (Emmelot *et al.*, 1959) ; hepatoma BY 225 has been described previously (Emmelot *et al.*, 1959).

Rat hepatoma BY 446.—The primary hepatoma was mainly of the adeno type ; the possibility might exist that part of this structure was due to lymphatic cysts which caused the surrounding tumour cells to attain an adenomatous appearance. Transplant generation 1 : mixed adeno-solid hepatoma ; transplant generations 2–20 : solid hepatomas containing cysts, the latter gradually disappearing so that a more uniform structure ensued ; transplant generation 20 : exclusively solid hepatoma, a few cysts ; transplant generations 22–26 : as 20, in one transplant of generation 26 more polymorphy and mitoses than in the others ; compare : Fig. 1 to 5.

Rat hepatoma BY 448.—The primary hepatoma was of the mixed solid-adeno type, lymph cysts were present. Transplant generation 1 : Only a few adenomatous structures, in part resembling those of the primary tumours. In this and the following transplants the character of the cells lining the tubules gradually gained the appearance of cells of the solid type and resembled less the polymorphic mucous producing cells of the cholangio type ; transplant generations 6–10 : no cysts,

uniform solid structure resembling the epithelial type of liver cells : transplant generations 11–20 : solid hepatomas, a few parts consisting of fusi-form (sarcoid) cells, adenomatous structures only very scarcely present, at some places trabecular arrangement of the cells but the latter was not characteristic for the whole tumour : compare : Fig. 6–10.

Rat hepatoma BY 463.—The primary tumour was the only one studied in the present series which was almost exclusively of the solid type. Some cysts, slight haemopoiesis and hyaline bodies were present. Upon transplantation no change in the overall structure occurred, a slight haemopoietic reaction was observed in the transplants but the hyaline bodies disappeared ; compare : Fig. 11 and 12.

Enzymic determinations.—Enzyme activities were studied in 105,000 × g supernatants prepared according to Pitot and Potter (1960) and Potter *et al.* (1960). Deoxycytidylate deaminase activity was determined according to Maley and Maley (1959) and Pitot and Potter (1960). [2-^{14}C] Thymine breakdown was studied using the system of Canellakis (1958) as modified by Potter *et al.* (1960). Enzyme activities were expressed in units, one unit standing for 1 μmole of deoxyuridylic acid formed or [2-^{14}C] thymine disappeared/amount of supernatant derived from 1 g. of fresh tissue/60 minutes at 37° C. The data listed in parentheses in Table II have been expressed per mg. of nitrogen of the soluble fraction.

RESULTS

Table I shows that the dCMP deaminase was completely missing from normal adult mouse liver and from the three transplanted mouse hepatomas selected for the present study. Two of the hepatomas, T 28012 and T 26473, showed negligible

EXPLANATION OF PLATES

(All slides 6 mμ, fixation Susa, stained with haematoxylin azophloxin.)

FIG. 1–5.—The BY 446 rat hepatoma. ×130.

FIG. 1–2.—Primary tumour : main pattern adenomatous, but combined with pseudo-adenomatous structures (lymphatic cysts ?) and solid trabecular parts (especially Fig. 2).

FIG. 3.—First transplant : adenomatous and solid.

FIG. 4.—Twentieth transplant : solid trabecular hepatoma throughout (some lympho-cysts). Rather uniform pattern, moderate amount of mitoses, some pleomorphism.

FIG. 5.—Twenty-sixth transplant : solid trabecular hepatoma as in Fig. 4, a few adeno-matous structures, conspicuous cyst formation, exhibiting more pronounced pleomorphism and mitotic activity than the other transplants 20–26 (see text—the present transplant contained 51 dCMP deaminase units). Other transplants (20–26) more like the one illus-trated in Fig. 4.

FIG. 6–10.—The BY 448 rat hepatoma. ×130.

FIG. 6.—Primary tumour : adenomatous part with inflammatory cells typical of this structure, mucus producing. Some haemopoiesis (not visible in this figure).

FIG. 7, 8.—First transplant : mainly solid with some adenomatous structures and solid trabecular parts forming slits of a regular (quasi-adenomatous) type.

FIG. 9.—Sixth transplant : solid hepatoma, shows slight slit formation lined by cells of the solid hepatoma type. At the right some quasi-adenomatous structures probably due to lining around infiltrated fat tissue.

FIG. 10.—Eleventh transplant : solid hepatoma, some necrosis and fibrosis ("old" transplant), increasing pleomorphism, slight fusicellular arrangement in some parts as illustrated here.

FIG. 11–12.—The BY 463 rat hepatoma.

FIG. 11.—Primary tumour (×130) : solid hepatoma of regular appearance, some cysts.

FIG. 12.—Third transplant (×340) : solid hepatoma with an overall structure resem-bling that of the primary tumour, slight haemopoiesis.

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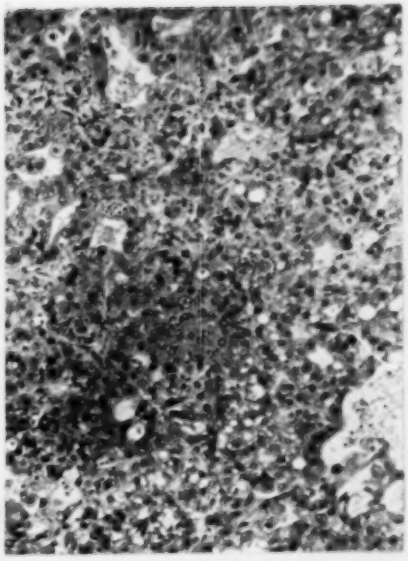

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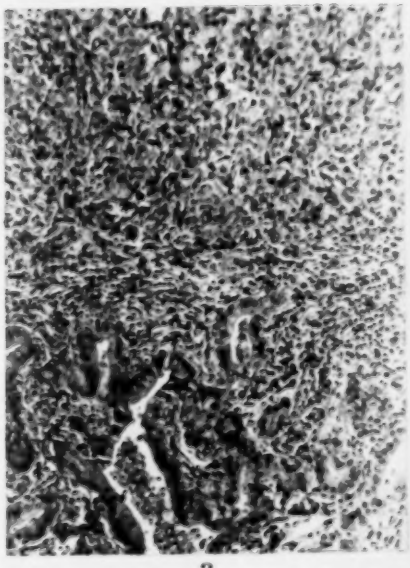

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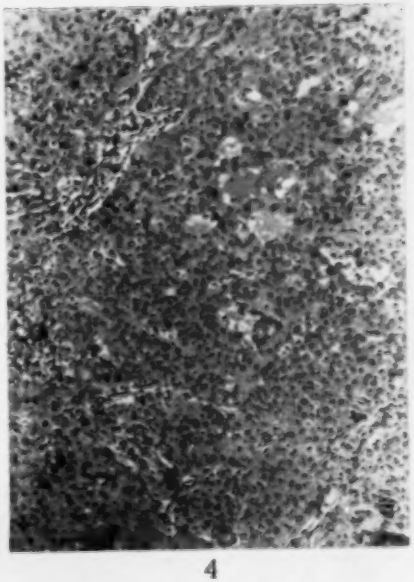

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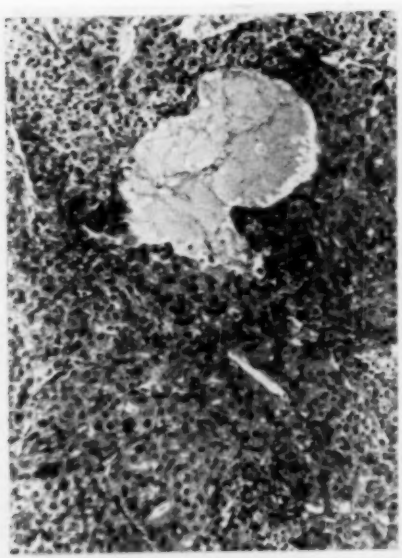
Emmelot, Hampe, Bos and Reyers.



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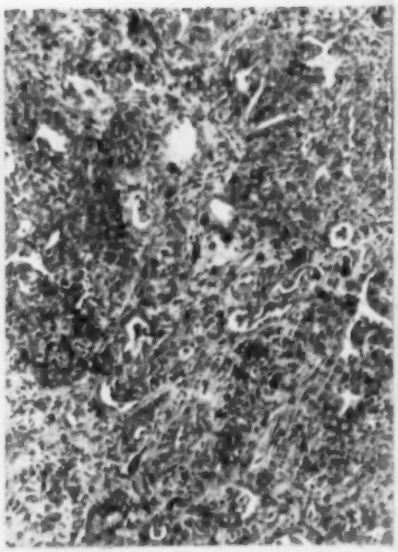

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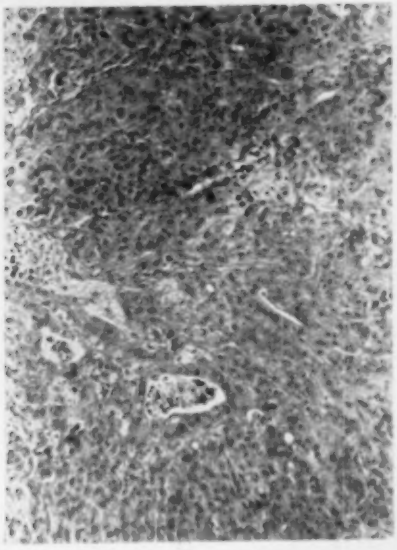

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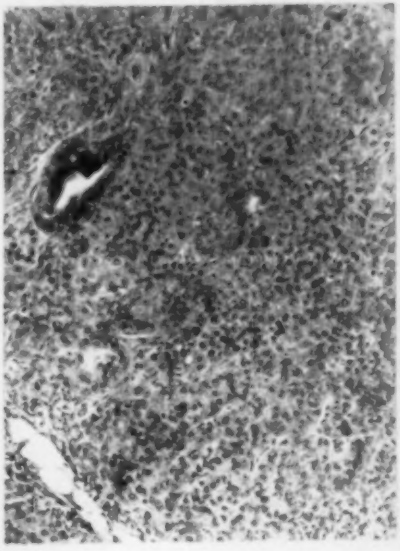

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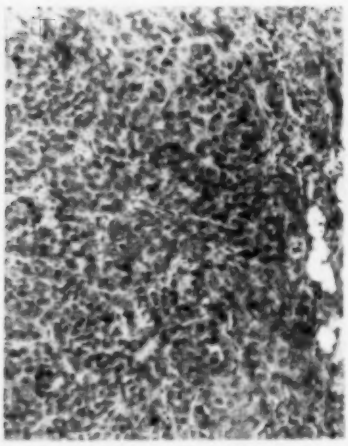

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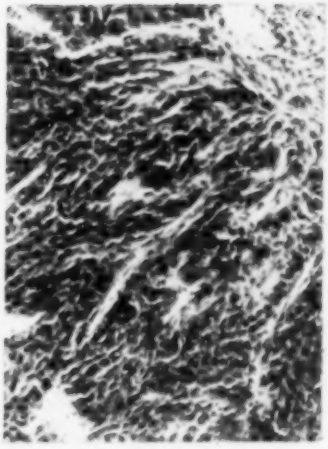

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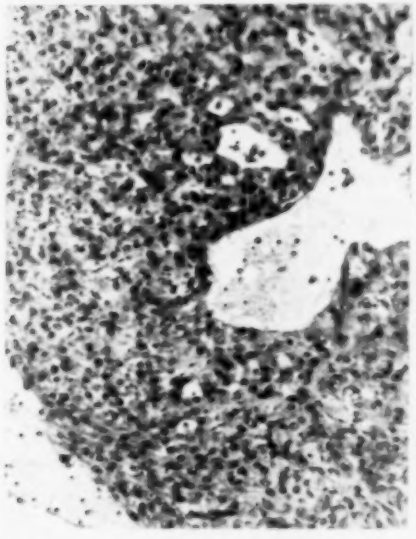

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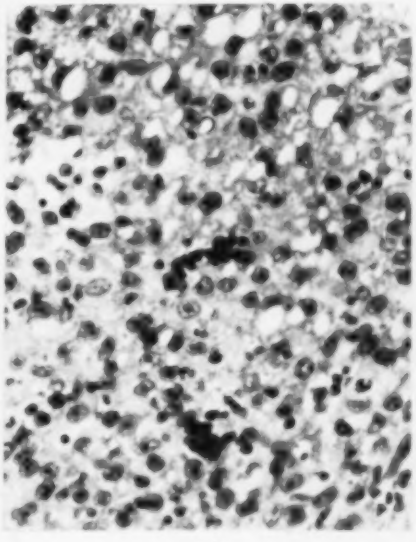

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activity in converting carbon atom 2 of thymine to carbon dioxide but hepatoma CBA 244 was about half as active as normal mouse liver. Adult rat liver appeared to be about twice as active as mouse liver but all four rat hepatomas studied possessed very little, if any, thymine catabolic activity. By contrast, the latter hepatomas showed various levels of dCMP deaminase activity.

TABLE I.—*Deoxycytidylate Deaminase and Thymine Catabolism in 105,000 × g Supernatants from Mouse and Rat Hepatomas*

Enzyme activity in units, as defined under Methods.

Tissue	Deoxycytidylate deaminase	Thymine catabolism
Mouse liver	0	1·15
Mouse hepatomas :		
T 28012	0	Insignificant*
T 26473	0	0
CBA 244	0	0·6
Rat liver	0	2·3
Rat hepatomas :		
BY 252	66–88†	Insignificant*
BY 463	70–77‡	,,
BY 446	8–81§	,,
BY 448	9–33§	0·36

* Five or less per cent of added radioactivity, [2–^{14}C] thymine, disappearing after 60 minutes, taken as experimental error.

† Transplant generation 102 : 11·2 μmoles deoxyuridylic acid formed/mg. N of soluble fraction/60 minutes.

‡ Transplant generation 7 : 8·0 μmoles deoxyuridylic acid formed/mg. N of soluble fraction/60 minutes.

§ Compare Table II.

It is of interest to compare the latter data with the histology of the hepatomas. The histological classification has been made in purely descriptive terms, the predicates solid (hepatocellular = parenchymous) or adenomatous (cholangiocellular) have been coined from analogy with the normal situation but do not necessarily refer to the cell origin of the hepatomas as stated in parentheses. This limitation is, of course, inherent to the histological discipline. This being so, it might not be astonishing to find a complete lack of correspondence between the histological classification and the dCMP deaminase levels of the hepatomas if the latter enzyme were to be a reliable marker for the cell origin of the tumours. A correspondence between the two sets of data, however, is by no means to be excluded on *a priori* grounds.

Two of the hepatomas, BY 225 and BY 463, both of an exclusively solid type, possessed high levels of dCMP deaminase activity, like those reported by Pitot and Potter (1960) for the Novikoff hepatoma ; one of these, BY 225, had been transplanted for many generations but the transplants of the BY 463 hepatoma had been carried through a few generations only. The description of these hepatomas as solid apparently does not imply that they were derived from parenchymal cells, since their enzyme levels were at variance with this assumption. Incidentally, neither do these results allow the enzyme levels to be correlated with the number of transplant generations through which the hepatomas had been carried, that is, with their growth rate which may be considered as an aspect of the actual stage of tumour progression.

Hepatomas BY 446 and BY 448, both also of the solid type, showed various levels of dCMP deaminase activity according to the number of generations through which they had been transplanted (Table II). The enzymic activities found in the

TABLE II.—*Deoxycytidylate Deaminase Activity of the Rat Hepatomas BY 446 and BY 448 in Relation to the Number of Generations through which the Transplants had been carried*

Enzyme activity as μmoles deoxyuridylic acid formed per equivalent of soluble fraction from 1 gram of fresh tissue (or per mg. N of soluble fraction, marked*) 60 minutes.

Transplant generation	Deoxycytidylate deaminase of Lepatoma : BY 446	BY 448
9	—	9
10	—	15
11	ε	30
13	27 (1·7)*	—
17	43 (3·5)*	—
20	81 (8·7)*	33 (2·7)*
22	21 (1·6)*	—
26†	44 (35–51) (4·9)*	—

† Several transplants of the 26th generation were studied, enzyme units ranging from 35 to 51 (4·1* to 6·0*).

transplants of BY 448 varied from low to moderate, whereas the activities of the BY 446 transplants showed a gradual increase (generations 11 to 20) followed by a decrease (generations 20 to 26). Since one or two transplants of a given generation were used for the enzymic assay, whereas the tumour line was maintained by another transplant of the same generation, an element of variability might have been introduced. The latter, however, does not appear to account for the differences observed in the enzymic activities of the various transplant generations of the BY 446 hepatoma (compare the data on the 26th generation listed in Table II and those mentioned under Materials and Methods). Moreover, such a variability as observed with the BY 446 transplants was not found in the case of the hepatomas BY 225 and BY 463 (Table II), whereas only a moderate increase in enzymic activity was observed with hepatoma BY 448 (Table II). The histological appearance of the various transplants used for the present enzymic determinations did not suggest that changes in the cells populations had occurred in respect to the relative contribution of adeno, dCMP deaminase positive, type of cells.

DISCUSSION

Mouse hepatomas

The classification of hepatomas as solid and of parenchymal origin is less subject to criticism as to the non-equivalence of these terms in the case of the mouse than in that of the rat. The absence of any dCMP deaminase activity from all three mouse hepatomas studied by us is in accord with what one may expect it to be if the enzyme is confined to bile duct cells.

As regards the thymine catabolizing enzymes, it is of interest that the hepatomas T 26473 and T 28012 take nearly four weeks to reach full size, whereas

hepatoma CBA 244 takes five weeks to do so. The finding that the latter hepatoma showed thymine catabolic activity whereas the two former did not, does not, therefore, contradict the concept (Potter, 1958, 1960; Potter *et al.*, 1960) that correlates the deletion of non-essential enzymes with the growth rate of the tumour. It should, however, be pointed out that this correlation does not necessarily indicate that an increased growth rate is the result of the deletion of these enzymes which are non-essential for cell division. The deletion might be part of a more complicated pattern of change, or even constitute merely a secondary symptom accompanying tumour progression, without being in the slightest sense responsible for the increase in malignancy. In this respect it remains of the greatest interest to ascertain to what extent the enzymes concerned with the catabolism of nucleic acid precursors do actually constitute a growth-regulator in normal tissue (Canellakis, Jaffe, Mantsavinos and Krakow, 1959; Hiatt and Bojarski, 1960).

Rat hepatomas

The results reported in Tables I and II allow the conclusion that no correlation between the histological data and the dCMP deaminase activities of the rat hepatomas can be established. Obviously, then, at least one of these two means of classification cannot be regarded as a reliable measure for the cell origin of the hepatomas. Either bile duct tumour cells grow in a solid pattern and are adenomatous structures in the hepatoma not equivalent, that is to say related, to similar histological structures in the normal liver, or else the dCMP deaminase activity is not an exclusive property of bile duct and tumour cells derived therefrom, while the enzyme might also be subject to adaptive or mutational events. Since the histological aspect can only be presented in descriptive terms which cannot be translated into direct relationships between the (normal and cancer) cells, whereas the enzymic data do reflect the functional aspects of the cell types, the latter are to be preferred as a criterion for the cell origin of the hepatomas, provided that certain conditions are satisfied. In the remaining part of this discussion we will be concerned with the latter question which, in our opinion, cannot as yet be answered in a positive sense.

The evidence that the dCMP deaminase is a genuine property of bile duct cells is only indirect and stems from the finding that feeding with a carcinogenic azo dye leads to positive dCMP deaminase activity in the liver (Pitot and Potter, 1960). However, apart from the induction of bile duct cell proliferation, the latter treatment also affects the parenchymal cells of the liver (Chang, Spain and Griffin, 1958; Porter and Bruni, 1959).

The finding of Pitot and Potter (1960) that embryonic liver does possess dCMP deaminase activity in contrast to the fully differentiated liver, consisting mainly of mature parenchymal cells, does not necessarily indicate that in the latter case the genetic information for the enzyme has been lost from the parenchymal cells. If only its cytoplasmic expression were to be suppressed as a result of the particular feed-back mechanisms operating in the normal adult liver, the potential information might be released again on the initiation of a new cytoplasmic state of metabolism accompanying the "mutational" conversion of a parenchymal cell to a tumour cell.

Such a phenomenon of physiological adaptation (or "modulation" at the enzymic level) might also come into operation at some time during the natural

history of liver tumour cells, either of parenchymal (enzyme : absent → present) or of bile duct cell (present → absent) origin, through an adaptation to progression.

A similar situation would exist if hepatoma cells were to be derived from the cells of a (hypothetical) stem layer in the adult liver, possessing the ability to differentiate to either one of the two cell types, and if such tumour cells retained at least partly, the latter capacity, while the actual expression of the specific properties might be subject to " modulation ". Thus, changes, perhaps of a partly reversible nature, might occur in the enzymology (and morphology) of liver tumour cells which one diagnoses arbitrarily as being either of parenchymal or of bile duct cell origin.

However, if the latter views are dismissed and the dCMP deaminase activity is regarded as a permanent property of bile duct, and not of parenchymal cells, and of the tumour cells derived therefrom, the absence of the enzyme in a hepatoma may still be due to a random mutation by loss in a bile duct tumour cell followed by selection. Changes in activity might be due to partial selection of new or pre-existing variants.

Since the specific activity of the enzyme per normal or cancerous bile duct cell is not known, positive dCMP deaminase activity in a hepatoma leaves one in doubt as to the presence of a mixed population of cells. Some tumour cells of bile duct origin with a " high " specific activity or many such cells with a " low " specific activity may be present, and this might vary from one hepatoma to another.

In the absence of further evidence, positive dCMP deaminase activity in a hepatoma, therefore, merely indicates that an unknown number of tumour cells of bile duct origin (if not of a *pseudo* bile duct type due to " modulation ") are present among a population of other tumour cells which are of parenchymal origin or of bile duct origin after having " lost " the enzyme. Even this conclusion may be complicated by the unknown dCMP deaminase activity of the stroma of the tumour. Histochemical methods may help to solve some of these problems. Such studies are being carried out at present (Willighagen and Planteydt, 1959), and it is hoped to publish the results in forthcoming papers together with electron microscopical observations and further biochemical features of the four rat hepatomas.

SUMMARY

Enzyme assays for deoxycytidylate deaminase and thymine catabolism were carried out on the soluble fractions from three transplanted mouse and four transplanted rat hepatomas. None of the mouse hepatomas, nor mouse liver, contained any deoxycytidylate deaminase activity but one of the mouse hepatomas, possessing a somewhat slower growth rate than the two others, was about half as active as normal mouse liver in converting carbon atom 2 of thymine to CO_2. Normal rat liver was twice as active as normal mouse liver in the latter respect, but the rat hepatomas showed very little, if any, activity. Thymine catabolism in relation to tumour progression is briefly discussed.

The four rat hepatomas carried moderate to high levels of deoxycytidylate deaminase activity. With one hepatoma marked variations were noted according to the number of generations through which the transplants had been carried. No correlation between the histology of the four hepatomas, which were predominantly of the solid type, and enzyme levels could be established. Though, generally

speaking, a functional classification of the rat hepatomas based on enzyme profiles should be preferred to a histological one which can be presented only in descriptive terms and does not necessarily refer to the cell origin of the hepatomas, the former procedure is beset with many difficulties. The latter are discussed and the conclusion is drawn that the deoxycytidylate deaminase cannot, as yet, be considered as a proper parameter of the cell origin of rat hepatomas.

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MEASUREMENT OF THE SULPHYDRYL CONTENT OF TISSUE CULTURES

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THE importance of sulphydryl (–SH) groups in the metabolism of cells and tissues has been under discussion for many years and estimations of tissue –SH have been carried out on numerous occasions. Absence of a method of –SH determination suitable for use on small samples, or on material of low –SH content, has limited the extent and value of such work. The development by Calcutt and Doxey (1959) of a method giving reasonably accurate and reliable –SH values on tissue samples of 50–200 mg. weight has made possible the determination of the amount of –SH present in the contents of a single tissue-culture bottle.

EXPERIMENTAL

Cultures used were of the HeLa cells and chick fibrocytes. The cells were washed off the walls of the culture bottles by means of a pipette, centrifuged down and twice washed with normal saline, being finally centrifuged to a compact mass. This was allowed to drain for a few minutes, weighed and the cells suspended in 50 ml. of 5×10^{-5} M *p*-chloromercuribenzoic acid (CMB) in 0·033 M phosphate buffer (pH 7·3). After 30 minutes standing to allow for penetration of the reagent into the cells and reaction with the –SH groups, the suspension was made up to 100 ml. with phosphate buffer and 25 ml. aliquots titrated against a 2×10^{-4} M solution of cysteine hydrochloride. The end-point was determined potentiometrically, using a transistorised instrument based on the design of Stock (1958).

For each batch of cysteine solution (prepared fresh each day) a control titration against the standard CMB was carried out.

RESULTS

Measurements of the –SH content of 40 samples of HeLa cells taken at 1–4 days after feeding gave a mean value of 10·1 $\pm$ 1·9 μg. per 100 mg. wet weight. On 10 samples of chick fibrocytes the mean value was 8·1 $\pm$ 2·6 μg. per 100 mg. wet weight.

Measurement of the –SH level at intervals of 1, 2, 3 and 4 days after feeding (feeding took place 7 days after sub-culturing) was carried out on 6 batches of HeLa cells. Results are shown in Fig. 1. The –SH level rises to a peak 2 days after feeding, returning to approximately the 1-day level on the 3rd and 4th days.

DISCUSSION

Table I shows the –SH content of HeLa and chick fibrocyte tissue culture cells and, for comparison, the –SH content of muscle from 11-day chick embryos and of some mammalian organs. All results were obtained by the method outlined above (Calcutt, and Doxey, 1959; Calcutt, Doxey and Coates, 1959; and unpublished work by these authors).

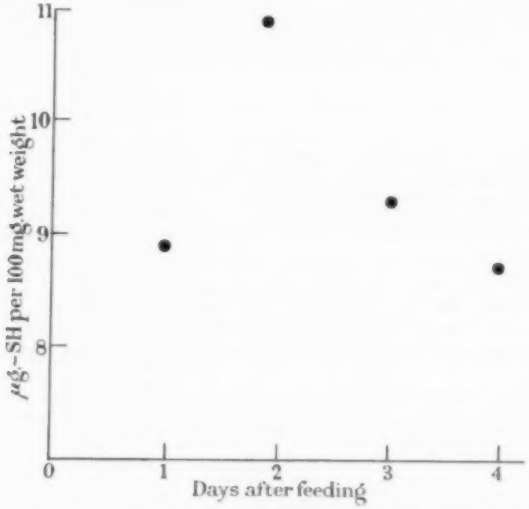

FIG. 1. –SH level in HeLa cells: Fluctuation with time after feeding.

TABLE I.—*Sulphydryl Content of Tissue Culture Cells and of Some Animal Tissues in μg –SH per 100 mg. Wet Weight*

Tissue cultures	
HeLa Cells . . .	10·1
Chick Fibrocytes . .	8·1
Chick embryo muscle .	3·1
Mouse thigh muscle .	12·1
Mouse liver (male) .	29·7

The –SH content of the tissue culture cells is lower than that of mouse thigh muscle, much lower than that of mouse liver and higher than that of muscle from 11-day chick embryos.

Chick fibrocytes and HeLa cells are maintained as actively growing tissue cultures, in which they are conducting all the functions of a complete organism. In 11-day chick embryo muscle, growth rate is very low and some functions are being carried out by other, specialised organs.

The differences in –SH level between chick fibrocytes and chick embryo muscle may perhaps be due to their different levels of physiological activity. Support for the idea that –SH level and degree of metabolic activity may in some way be

correlated, comes from the very high –SH level in mouse liver, which is known to be the seat of intense biochemical activity, and from the fluctuations of liver –SH level in mice which have been injected with polycyclic hydrocarbons, which are known to be metabolised in the liver (Calcutt, Doxey and Coates, 1959). That –SH groups are essential for the activation of many enzymes (Elliot, 1946) lends further support to this view.

The rather higher –SH content of mouse thigh muscle might be accounted for by the greater metabolic activity of the muscle of an active mouse (although growth has ceased) than in the inactive chick embryo.

However, the limited reliable information available on the amount and distribution of sulphydryl in tissues precludes the drawing of any more definite conclusions at present.

SUMMARY

The sulphydryl content of 40 samples of the HeLa cells, measured by the method of Calcutt and Doxey (1959) was 10·1 $\pm$ 1·9 μg. per 100 mg. wet weight. For 10 samples of chick fibrocytes the –SH content was 8·1 $\pm$ 2·6 μg. per 100 mg. wet weight.

The author would like to thank Dr. A. K. Powell and his assistants for providing the HeLa and chick fibrocyte tissue cultures used in this work.

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FURTHER STUDIES OF THE EFFECTS OF CHEMICAL CARCINOGENS UPON THE –SH LEVELS OF TARGET AND NON-TARGET TISSUES

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An examination of the effects of several chemically unrelated carcinogens upon the sulphydryl (–SH) levels of various susceptible and non-susceptible tissues led to the question of an increase in –SH levels being an essential feature of tumour induction (Calcutt, Doxey and Coates, 1960). This is an obviously important issue and so further evidence has been sought. A series of different chemical carcinogens has been tested as previously and the detailed findings are given below.

The measurement of tissue –SH

The technique employed was the same as in the earlier work. Full details are given by Calcutt and Doxey (1959) and Calcutt *et al.* (1960).

EXPERIMENTAL

o-Aminoazotoluene

This is a powerful hepatocarcinogen in mice and will also induce lung tumours, but as far as the data recorded by Hartwell (1951) go, it does not appear to cause kidney tumours. A batch of Strong A male mice were each given a single subcutaneous injection of 8 mg. of *o*-aminoazotoluene in 0·25 ml. of olive oil, this being a dose and route found by Andervont (1947) to be effective in eliciting both liver and lung tumours. Fifteen mice from the same original lot were taken as controls.

At intervals over a period of ten weeks the control animals were killed and the –SH levels of liver, kidney and lung were measured. Corresponding measurements were also made in respect of the experimental animals, these being killed singly daily for four days and then at weekly intervals over the period of ten weeks.

The results for the liver are shown in Fig. 1. Since no pattern was discernible in the figures for the control animals these have been plotted as a mean and standard deviation. It will be seen that the –SH level of the experimental livers rose above the control figure 48 hours after commencement of the experiment and then remained persistently high. In this and all subsequent experiments –SH levels are expressed as μg. of –SH per 100 mg. wet weight of tissue.

The findings for lung tissue are shown in Fig. 2. Here again the control figures are shown as a mean and standard deviation. The experimental figures

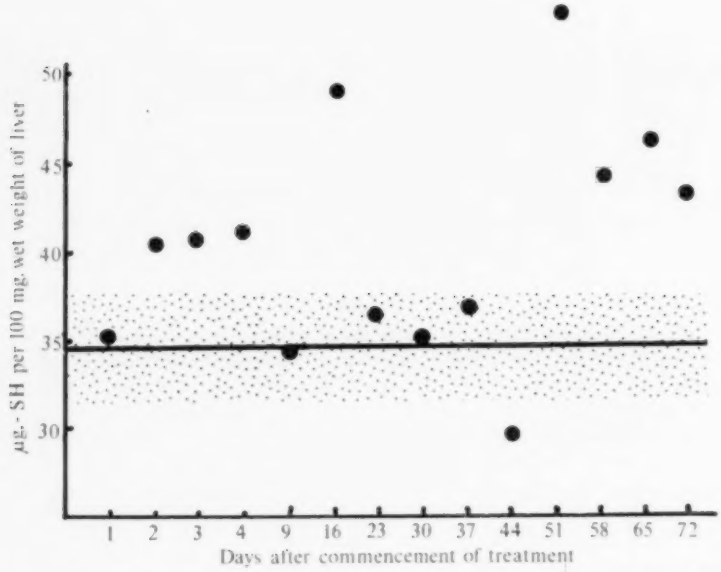

FIG. 1.—The effects of *o*-aminoazotoluene on mouse liver –SH values. In this and all succeeding figures the mean control value is shown as a heavy continuous line and the extent of the standard deviation is indicated by the dotted area.

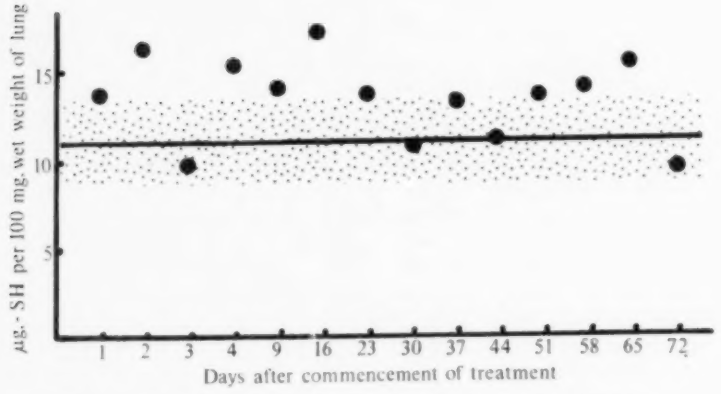

FIG. 2.—The effects of *o*-aminoazotoluene on mouse lung –SH values.

tend to be above the control level and in a few instances show a considerable elevation.

The kidney results are shown in Fig. 3. Here there is no apparent distinction between the control and experimental values.

Carbon tetrachloride

Given in repeated small doses this agent induces hepatomas in mice. A group of mice were each given 0·005 ml. of carbon tetrachloride twice a week. The carbon tetrachloride was emulsified in distilled water with an Atomix blender

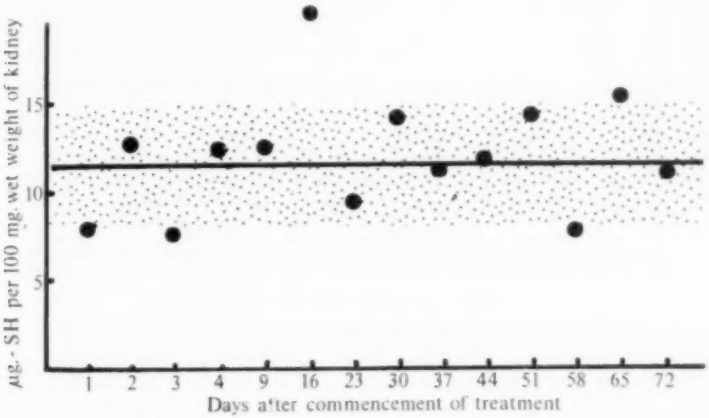

FIG. 3.—The effects of *o*-aminoazotoluene on mouse kidney –SH levels.

and given as 0·5 ml. of emulsion by intraperitoneal injection. The control animals used in the previously recorded experiments with *o*-aminoazotoluene also served as controls in this experiment, since all animals were taken from the same original stock and the experiments were performed concurrently. The –SH levels for liver (target tissue) and kidney (non-target tissue) were determined daily for four days and then weekly for up to ten weeks. The results for the liver estimations are shown in Fig. 4. Between the third and eighth weeks of treatment the level in the livers of the treated animals was found to rise well above the mean control value.

The kidney results are shown in Fig. 5. Here no distinction could be found between control and experimental values.

1,2 : 5,6-*Dibenzacridine*

This agent is a weak carcinogen with a long latent period when tested on mouse skin. In the present work this compound has been painted once weekly as a 0·3 per cent solution in acetone on the backs of a series of Strong A female mice. A similar group of mice acted as controls. Measurements of skin –SH levels of both controls and experimentals were made at intervals over a period

of 120 days. The results are shown in Fig. 6. There is a general tendency for the level in the experimental skins to be a little above that of the control series.

In this particular experiment no other tissues were examined.

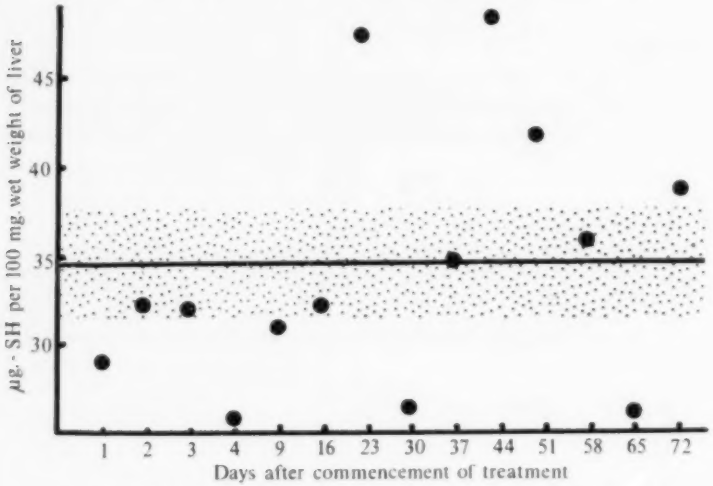

FIG. 4.—The effect of carbon tetrachloride on mouse liver –SH levels.

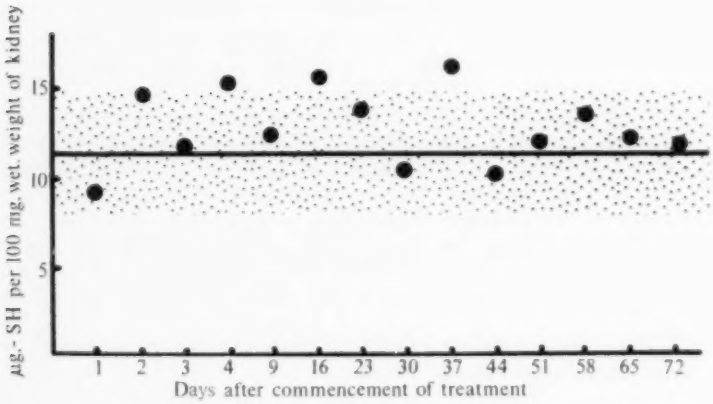

FIG. 5.—The effects of carbon tetrachloride on mouse kidney –SH levels.

p-*Dimethylaminoazobenzene*

Previously Calcutt *et al.* (1960) found that when this agent was fed at the rate of 0·6 per cent of the diet to rats on a poor quality diet there was an elevation of liver –SH levels for a limited period. This agent has now been retested against

rats on a high protein/high vitamin diet, i.e., under conditions where tumour formation would not be expected. The dyestuff was fed at the rate of 0·6 per cent of the diet to a group of rats, whilst a similar group on the same diet, but

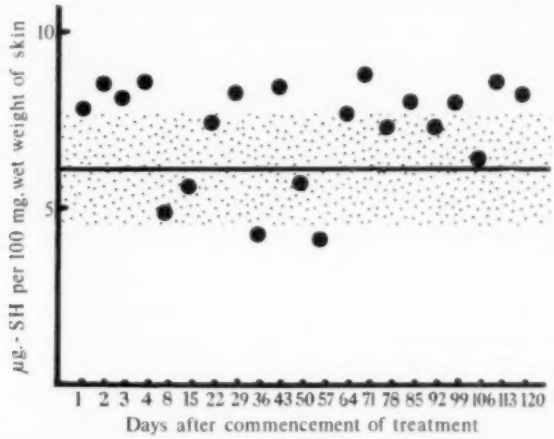
10
5
μg. - SH per 100 mg. wet weight of skin
1 2 3 4 8 15 22 29 36 43 50 57 64 71 78 85 92 99 106 113 120
Days after commencement of treatment

FIG. 6.—The effects of 1,2 : 5,6-dibenzacridine on mouse skin –SH levels.

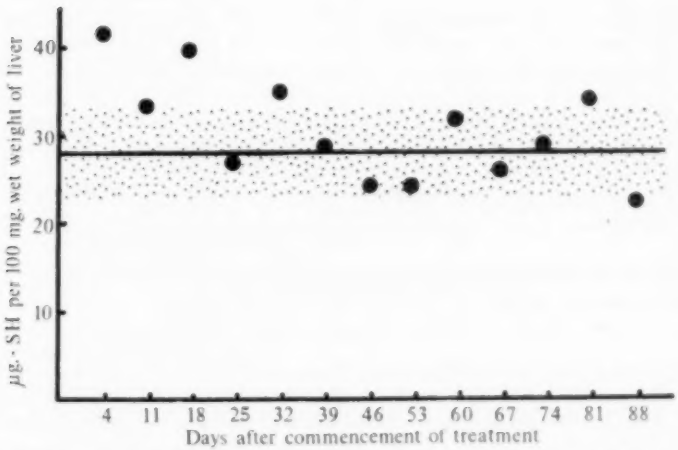

FIG. 7.—The effects of *p*-dimethylaminoazobenzene on the liver –SH levels of rats on a good quality diet.

without the dyestuff, acted as controls. Liver and kidney –SH levels were estimated on both control and experimental animals over a period of twelve weeks.

The findings in respect of the liver are shown in Fig. 7. It is immediately apparent that there is no distinction between control and experimental levels.

The results for the kidneys are shown in Fig. 8. The –SH level of the experimental kidneys is persistently a little higher than that of the controls.

DISCUSSION

The experimental results reported above fall into line with those previously recorded by Calcutt *et al.* (1960). In the earlier work the most obvious effect was obtained with the powerful carcinogen : 3,4-benzopyrene, whilst weaker agents showed lesser effects. The present work has shown the powerful agent, *o*-aminoazotoluene, to have a very well defined effect on the liver of mice and a lesser effect upon the lung tissue of the same animals. Under the conditions of dosage and route used *o*-aminoazotoluene would be expected to produce a high yield in the lungs.

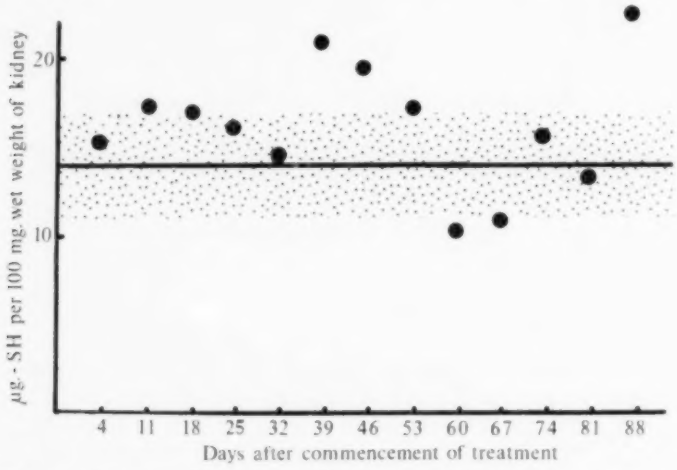

FIG. 8.—The effects of *p*-dimethylaminoazobenzene on the kidney –SH levels of rats on a good quality diet.

Carbon tetrachloride has been found to cause an elevation of liver –SH levels in mice. Since this agent also causes fatty infiltration of the liver and all results have been calculated on the basis of wet weight of tissue the experimental results in this series will be effected by the presence of fat in the tissue. In fact, if allowance were made for this fat and the results were calculated as against weight of active liver tissue, the experimental figures would be higher than those recorded.

A very interesting set of figures in the present series is that obtained with *p*-dimethylaminoazobenzene in animals on a high quality diet. There was no distinction between the experimental and control groups, whereas earlier it was found that in animals on a poor (tumour promoting) diet there was a period when the livers of the experimental animals showed a higher –SH level than did the control livers. This suggests that with this agent any tissue –SH elevation is a

consequence of dietary factors and is not due to the agent *per se*. Some evidence for this resides in the finding by Calcutt, Doxey and Coates (1961) that deletion of riboflavin from animals causes an elevation of liver –SH levels.

If the present results are taken together with those previously obtained by Calcutt *et al.* (1960) we have five different chemical carcinogens (3,4-benzopyrene, urethane, 2-fluoroenylacetamide, *o*-aminoazotoluene and carbon tetrachloride) which cause an elevation in the –SH levels of target tissues during the tumour induction period. None of these agents produced a similar –SH rise in non-target tissues. Similar, but not so well defined, results were obtained with *p*-dimethylaminoazobenzene, 1,2 : 5,6-dibenzacridine, and stilboestrol. To this can be added the finding by Boyland and Mawson (1938) that 1,2 : 5,6-dibenzocarbazole (a hepato-carcinogen) induces a large and prolonged rise in liver glutathione, whilst methylcholanthrene and 1,2 : 5,6-dibenzanthracene (neither affects the liver) had no such effect.

On the basis of the above data it is now possible to offer a tentative hypothesis in respect of the induction of tumours. It is already well recognised that tumour induction is a multi-stage process. It is now suggested that, apart from the agent causing initial cell damage, a necessary requirement for a tumour to appear is the elevation of the tissue –SH level above the normal level of the particular tissue. This elevation could arise directly as the result of application of the carcinogen as with 3,4-benzopyrene or *o*-aminoazotoluene or indirectly, as with *p*-dimethylaminoazobenzene where dietary restriction is necessary for tumour induction.

This hypothesis also offers a reasonable explanation for many findings in respect of anticarcinogens. Crabtree (1944, 1945, 1946) found that mercapturate forming agents and various other compounds known to inhibit –SH groups acted as anticarcinogens in respect of the induction of skin tumours by polycyclic hydrocarbons. This activity was believed to reside in the fact that combination of the hydrocarbon with tissue –SH groups was an essential for tumour formation and that prevention of this reduced the chance of a tumour occurring. There has, however, never been any substantiation of the idea of hydrocarbon/–SH binding. It can now be suggested that these anticarcinogens are active in that they inhibit an essential rise in tissue –SH levels.

So far the evidence in favour of the above views is little more than fragmentary, but at least the hypothesis offers a basis for further experimental work. Investigations of some further aspects of this problem are already in hand and will be reported shortly.

SUMMARY

o-Aminoazotoluene has been found to induce a rise in –SH levels in mouse liver and lung but not kidney. Carbon tetrachloride causes an elevation of mouse liver –SH levels but not those of kidney.

1,2 : 5,6-Dibenzacridine causes a slight rise in mouse skin –SH levels.

p-Dimethylaminoazobenzene does not affect mouse liver –SH levels if the animals receive a high quality diet.

On the basis of this work and other evidence in the literature it is suggested that an elevation of tissue –SH levels is an essential prerequisite for tumour formation.

The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

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THE INFLUENCE OF THIAMINE AND PANTOTHENATE UPON TISSUE –SH LEVELS

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As a result of studies of the sulphydryl (–SH) levels of various animal tissues which had been subjected to chemical carcinogens, Calcutt, Doxey and Coates (1961*a*) have suggested that a rise in tissue –SH level is an essential prerequisite for tumour formation. It has also been shown that riboflavin deletion leads to a rise in liver –SH levels, whilst the vitamin in the diet or by injection will reduce –SH levels (Calcutt, Doxey and Coates, 1961*b*). This fact would appear to be related to the known protective action of riboflavin against azodye carcinogenesis. Deletion of riboflavin from the diet was also found by Russell (1945) to halve the induction time of brain tumours in rats treated with methylcholanthrene. At the same time Russell also found that thiamine deletion reduced the induction time for similar tumours.

This finding suggests the possibility that thiamine could also affect –SH levels in a similar fashion to riboflavin. Support for such a suggestion derives from the finding by Hsu and Chow (1960) that the liver glutathione levels of rats on a thiamine deficient diet are much higher than those of animals on normal diets. A possible mechanism for thiamine affecting –SH levels is suggested by the conclusion of Metcalf (1943) that thiamine or pantothenate will, in the cockroach, *Periplaneta americana*, cause an *in vivo* release of bound riboflavin to free tissue riboflavin.

Further evidence for thiamine and pantothenate affecting tissue –SH levels has, therefore, been sought. For this work mice have been used as the animals, and total tissue –SH levels have been estimated by the method described by Calcutt and Doxey (1959) and Calcutt, Doxey and Coates (1960).

EXPERIMENTAL

Effects of vitamins by injection.—A batch of thirty Strong A female mice were divided into three groups of ten. One group acted as controls whilst the animals of another group each received one intraperitoneal injection of 0·1 mg. of thiamine hydrochloride in 0·5 ml. of distilled water. The animals of the third group each received a single intraperitoneal injection of 0·1 mg. of calcium pantothenate in 0·5 ml. of distilled water. Estimations of the –SH levels of the livers of the control animals were made and a mean control figure and standard deviation calculated. The experimental animals were killed at half hourly intervals and the liver –SH values measured.

The results for the animals treated with thiamine hydrochloride are shown in Fig. 1 and those for the experiment with pantothenate in Fig. 2. In both cases the liver –SH values fell to well below the mean control values.

In a further experiment similar groups of female mice were set up as previously, but this time the experimental animals were killed at daily intervals, commencing 24 hours after the time at which the injection had been given. Neither experimental series showed any distinction from the control series, indicating that the effect on liver –SH values was of less than 24 hours duration.

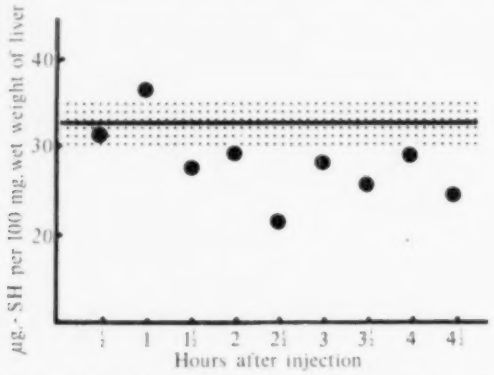

FIG. 1.—The effect of thiamine hydrochloride on mouse liver –SH values. The mean control value is shown as a heavy line and the extent of the standard deviation is indicated by the dotted area. Experimental points are shown as filled circles.

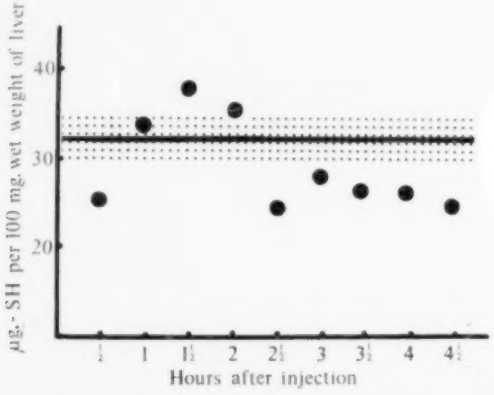

FIG. 2.—The effect of calcium pantothenate on mouse liver –SH values. The mean control value is shown as a heavy line and the extent of the standard deviation is indicated by the dotted area. Experimental points are shown as filled circles.

Effects of deletion of the vitamins.—A batch of forty Strong A male mice was transferred to a synthetic diet obtained as a vitamin free diet from British Drug Houses, Ltd., London. Vitamins were added to this in the amounts used by Greenstein, Otey, Birnbaum and Wintz (1960) in their experiments on synthetic diets. After a period of three weeks on this diet the animals were divided into a group of ten as controls and two groups of fifteen as experimental animals.

The controls received the same diet as previously, one experimental group received the diet minus thiamine and the other group the diet minus pantothenate.

The animals were killed singly at daily intervals and estimations of liver and skin –SH levels were made. The results for the thiamine deficient animals are shown in Fig. 3 (liver) and Fig. 4 (skin) and the corresponding data for the panto-

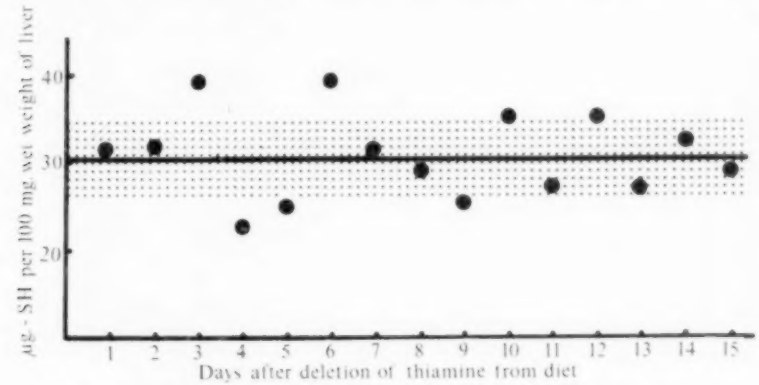

FIG. 3.—The effects of deletion of thiamine on mouse liver –SH values. The mean control value is shown as a heavy line and the extent of the standard deviation is indicated by the dotted area. Experimental points are shown as filled circles.

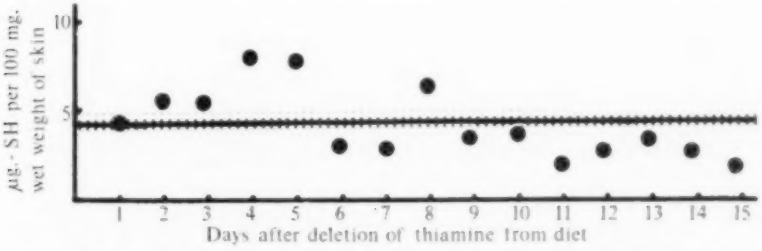

FIG. 4.—The effects of deletion of thiamine on mouse skin –SH values. The mean control value is shown as a heavy line and the extent of the standard deviation is indicated by the dotted area. Experimental points are shown as filled circles.

thenate deficient animals are given in Fig. 5 (liver) and Fig. 6 (skin). In both experimental series the skins showed a small elevation in –SH level for a short period after commencement of the experiment and then declined to below normal values. The livers showed little effect initially and then tended to fall below normal values.

DISCUSSION

The results obtained in the present experiments are not very striking, although they do indicate that thiamine or pantothenate levels can affect tissue –SH levels. This is in contrast to the previously found effects with riboflavin, where the effect was of considerable magnitude, of rapid onset and long duration (Calcutt, Doxey

and Coates, 1961*b*). The slight effects found in the current work could be consistent with an explanation based on the release of bound riboflavin (this being the active agent) as found by Metcalf (1943) in his experiments with cockroaches.

It is interesting that one of the major sites of physiologic activity of thiamine is the central nervous system, and it was in experiments on nervous tissue that

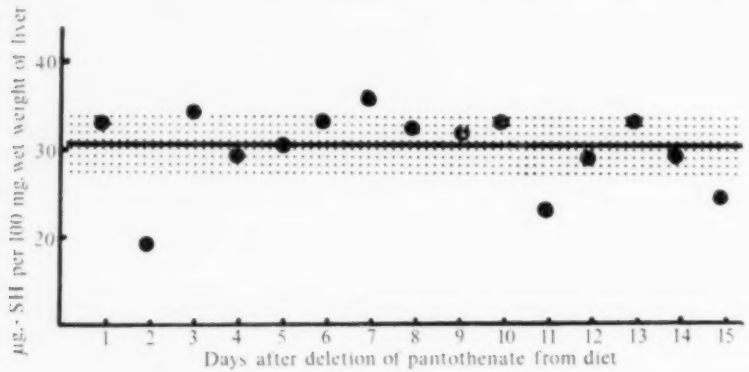

FIG. 5.—The effects of deletion of pantothenate on mouse liver –SH values. The mean control value is shown as a heavy line and the extent of the standard deviation is indicated by the dotted area. Experimental points are shown as filled circles.

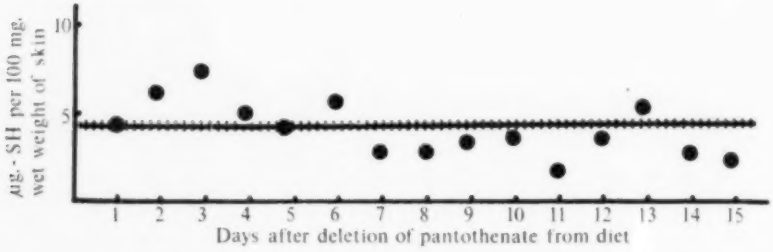

FIG. 6.—The effects of deletion of pantothenate on mouse skin –SH values. The mean control value is shown as a heavy line and the extent of the standard deviation is indicated by the dotted area. Experimental points are shown as filled circles.

Russell (1945) achieved effects on tumour induction with thiamine deficiency. Whether nervous tissue would react to thiamine deletion in the same fashion as skin cannot be said at the moment, but it may not be without point that embryologically the nervous system has a common origin with skin.

If the suggestion of Calcutt, Doxey and Coates (1961*a*) that a rise in tissue –SH levels is an essential prerequisite for tumour induction be accepted then the present results fall into place. The fact that thiamine or pantothenate levels have only minor effects on tissue –SH levels is reflected in the general lack of reports of effects of these vitamins on tumour formation.

SUMMARY

Thiamine hydrochloride or calcium pantothenate by intraperitoneal injection cause short lived decreases in liver –SH values.

Deletion of the vitamins from the diet cause temporary increases in skin –SH levels but have little effects on liver –SH values.

These findings are briefly discussed in relation to tumour induction.

The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

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DEOXYRIBONUCLEIC ACID CONTENT OF MOUSE ASCITES TUMOUR CELLS IN INTERPHASE AND MITOSIS

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THE distribution of chromosome numbers and cellular deoxyribonucleic acid (DNA) values in both human and animal neoplasms show that those studied to date conform to the concept of the stem cell in tumour cell proliferation. The growth of a tumour depends on the progressive multiplication of its stem cells which form the majority of the cell population. Their genotype is characterised by their chromosome complement or DNA content ; this is not necessarily euploid.

However, in addition to the stem cells, there are often other neoplastic cells present with a degree of ploidy different from that of the stem line. Their frequency varies from tumour to tumour, and their chromosome number or level of DNA may exceed that of the stem cells, sometimes very considerably. Such cells do not appear to be associated with any particular stage of tumour development, and their continued presence in experimental neoplasms throughout numerous transplantations raises the question of their mode of origin. The possibilities are that they propagate by continued normal mitosis following their original appearance in the cell population, or that they arise *de novo* at intervals from the stem cells, and fail to reproduce.

The problem is not one of academic interest only, since the repeated clinical observation on human tumours of an initial satisfactory response to irradiation, chemotherapy or hormone treatment followed by subsequent resistance and renewed growth, points to the need for a more detailed knowledge of the genesis of the newly-resistant cell population.

At first sight the existence of tumour cells other than those of the stemline suggests a potential reservoir from which a new resistant strain might arise should it be favoured by the selective action of environmental alterations due to therapeutic measures. If so, the least likely candidates in the circumstances are the giant cells encountered from time to time, and one may doubt whether such cells are capable of giving rise to viable progeny at all. However, the status of those cells differing by only a relatively minor degree in ploidy level from that of the stem cells is worth further consideration although it may well be that the change involved is related to a point mutation only.

This paper describes the changes in distribution of cellular DNA values during interphase and at different stages of mitosis, in a mouse ascites-cell tumour, indicating that those cells with markedly increased DNA content do not reproduce themselves, but presumably arise as a result of mitotic aberrations, some of which are also responsible for aneuploidy.

MATERIALS AND METHODS

Smears were made of a mouse ascites-cell tumour (T.2146), grown in an inbred laboratory strain of albino mice, taken at various times following transplantation. These were fixed immediately in methanol, stained with Feulgen, and microdensitometric measurements were then carried out on cells selected at random as described previously (Meek, 1960), using normal leucocytes as a standard of reference.

Estimations of DNA content, as judged by the absorption level, were made on cells in the intermitotic phase, and in metaphase, anaphase and telophase, taking 150 in each group, with anaphase and telophase counted together as one group.

RESULTS

The distribution of cellular DNA content during interphase and in mitosis is seen in the histogram (Fig. 1). It is apparent that there is a considerable degree of both aneuploidy and polyploidy, and the wide range of values of nuclear DNA content is reflected in the microscopical appearances of the Feulgen-stained nuclei in smears.

These features are, however, common to tumours in general. The additional information gained in this study by microdensitometry is, first, that in spite of the wide scatter of cell types as shown by their DNA content, the cell population is dominated by the presence of a stem-cell line on which the growth of the tumour depends, and second that the polyploids in the higher ranges fail to divide.

Judged by the degrees of absorption in interphase, the major mode of DNA content lies between diploid and tetraploid levels. There is nevertheless a considerable proportion of the cells spread between tetraploid and octoploid values. Out of the 150 cells in interphase 39 (26 per cent) have DNA values higher than octoploid.

In metaphase, the major mode lies between tetraploid and octoploid levels, in a position corresponding to about twice the DNA content of the stem cells as shown by the interphase readings. Here again there is a very wide range of distribution of cellular DNA content with a quarter of the population beyond the octoploid level. Since the stem-cell line is appreciably below the tetraploid value this indicates the extent of polyploidy in the tumour. Of these, 17 have a DNA content greater than that of 12-ploidy.

The anaphase-telophase histogram shows a striking alteration in distribution. Ninety-three per cent of all these cells are situated in the diploid-to-tetraploid zone, and the major mode representing the stem cells is prominent ; this is in about the same position as in interphase. The remaining 7 per cent lie in the tetraploid-to-hexaploid region ; none exceed the hexaploid value.

DISCUSSION

Variations in the amount of absorbing material present in single cells may be due to alterations in the DNA-Feulgen reaction, errors in measurement, synthesis of DNA during interphase, or to changes in the basic cell content of DNA with or without an alteration of the number of chromosomes.

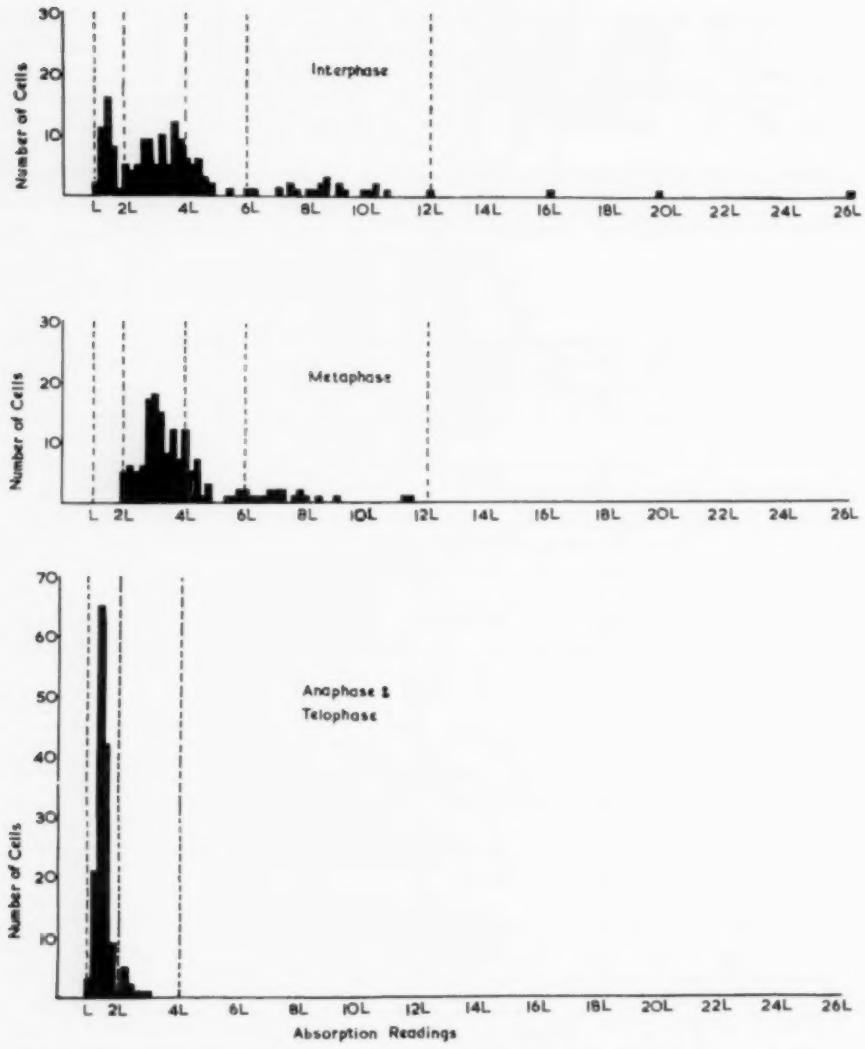

FIG. 1.—Absorptiometric readings representing DNA content in 150 cells in interphase, 150 in metaphase, and 150 in anaphase and telophase combined. In the latter group, the readings are those taken from the two separated masses of DNA.

With regard to the first factor, it is not known whether variations take place in the stoichiometry of the Feulgen-DNA complex in these cells during mitosis; this might be investigated by parallel measurements of the absorption of ultraviolet light on individual cells. As far as possible the errors inherent in microabsorptiometry have been minimised by the use of the integrating microdensitometer designed by Deeley (1955).

In interphase, doubling of the amount of DNA occurs preparatory to mitosis and this leads to a spread in the pattern of cellular distribution, which is not related to genotypic differences. The wide distribution of readings in interphase is undoubtedly due in part to stem cells which had already completed their premitotic synthesis of DNA or were in the process of doing so. The results obtained in this study from the cells in interphase show a prominent mode surrounded by an extensive scatter of DNA values. The mode is taken to represent the stem cells responsible for the growth of the tumour, in accordance with the stem-cell concept of proliferation put forward by Hauschka and Levan (1953) and Makino and Kano (1953). In metaphase the mode is seen between tetraploid and octoploid values, in a position which agrees well with that expected from a doubling of the interphase stem-cell value. The metaphase content is then halved in normal mitosis when the cells proceed to the later stage, and here in anaphase and telophase the stem line is in about the same position as in interphase. Clearly the interphase group contains a large number of post-telophase cells which had not started synthesis of DNA. In view of the high mitotic rate it is interesting that there is a sufficient number of post-telophase cells to indicate the stem cell mode so clearly. These cells are responsible for the proliferation of the tumour.

In addition to the stem cells this tumour contains a number of aneuploids and polyploids. The presence of the former is suggested by the wide spread of values around the stem-line.

In both interphase and metaphase a quarter of the cell population is represented by DNA values exceeding the octoploid level, one cell in interphase having a DNA content equivalent to a state of 52-ploidy. The scatter between octoploid and 24-ploid levels is similar in these two stages, and one might expect that if the cells seen in metaphase completed mitosis, there would be a similar extension of readings in anaphase and telophase up to 12-ploidy. This is not so however, and the values in the latter group end at hexaploidy. The 17 metaphase cells with values greater than 12-ploidy are therefore not represented at all in the later stages of mitosis. However, those metaphase cells (22) with values in the region between octoploidy and 12-ploidy are reflected in the 10 cells in anaphase and telophase lying between tetraploid and hexaploid levels. The results suggest a failure on the part of the cells with high DNA content to continue in mitosis beyond metaphase. This failure appears to be relative to the level of ploidy, for those cells in the lower range of polyploidy evidently may enter anaphase or telophase, although apparently in fewer numbers, whereas those with still higher contents of DNA do not appear to proceed beyond metaphase at all.

It will be observed that there are three cells in interphase with a very high DNA content; such giant cells are encountered occasionally in this tumour. None of corresponding size were seen amongst the metaphase cells. It is impossible to draw any firm conclusions from this observation alone as to whether these may enter mitosis or not, although one may doubt whether such grossly

abnormal cells do so. Since, however, some polyploids at the lower levels do not continue in mitosis beyond metaphase, and since those which do so appear to decrease in number as the level of ploidy increases, it may be questioned whether those of still higher degrees of polyploidy even reach metaphase.

How did these polyploids arise, and what is their fate if they fail to pass the stage of metaphase?

Polyploids may occur as a result of endoreduplication, endomitosis and other forms of reduplication. Levan and Hauschka (1953) reported endoreduplication in mouse ascites tumour cells, with increase of ploidy taking place during interphase in the absence of nuclear changes other than expansion of nuclear volume. If such cells subsequently enter mitosis diplochromosomes are seen in the ensuing metaphase. In another study of the present tumour using aceto-orcein preparations, diplochromosomes were seen very occasionally, so it seems that interreduplication takes place in this material and accounts for some of the polyploids. If these cells with a high content of DNA fail to undergo cleavage they may either reconstitute their chromosomes into a nucleus without entering anaphase and thus return to interphase, or they may degenerate.

At first sight the metaphase population appears to include, apart from stem cells and polyploids, a large percentage of aneuploids. Richards, Walker and Deeley (1956) cite the proposition that the metaphase distribution of ploidy reflects the degree of aneuploidy in a cell population. They found, however, that in Krebs and Ehrlich mouse ascites cell tumours, their results showed less aneuploidy in interphase than expected on this basis from the state in metaphase, and they suggested that aneuploid cells may linger in metaphase so having an unduly marked effect on the pattern of distribution of DNA content at this stage. Hsu and Moorhead (1956) followed mitosis in HeLa cells by time-lapse cine sequences of the cultures, and reported that metaphase may last many hours in some of the cells. If a similar prolongation of metaphase occurs in the tumour studied here, then the degree of aneuploidy may not be as great as would appear from the metaphase histogram.

As to the origin of the aneuploids, these may arise from multipolar spindles and other mitotic aberrations leading to asymmetrical division. The completion of division of cells with multipolar spindles has been reported by Hsu and Moorhead (1956) to take place in cultures of HeLa cells. Variations in microenvironment may tend to have a selective influence on the cell genotypes, so that one type would be favoured in a given situation more than another. In a solid tumour, conditions may alter slightly from one area to another, and this would exaggerate the heterogeneity of the tumour cell population. However, in an ascites-cell tumour, the environmental conditions are the same for all cells, so that this factor does not influence the extent of genotype variations seen amongst the cells.

In the present case it will be noted that, whilst the great majority of cells seen in anaphase and telophase can be assigned to the stem line by reason of the type of DNA distribution, there is also a number of heteroploids which appear to be successfully completing mitosis. It is clear, however, that the main growth of the tumour depends on multiplication of the stem cells, and so the pattern of cell proliferation in this case can be said to conform to the stem-cell concept.

The inheritance of an unstable mitotic mechanism is a common feature of neoplasms leading to the continued appearance of aneuploids and polyploids, as

in this tumour. These results suggest that the more their cellular DNA content is increased above that of the stem cells, the less likely are they to reproduce themselves.

SUMMARY

Measurements of DNA content were carried out by microdensitometry on mouse ascites tumour cells in interphase, metaphase, anaphase and telophase.

The modal value representing the stem cells was between diploid and tetraploid levels in interphase, anaphase and telophase.

A considerable population of polyploids was present in interphase and metaphase. Those in the upper range were not represented in the anaphase and telophase distribution, and evidently do not divide. Those with moderately increased levels of ploidy were found in anaphase and telophase, but their percentage representation in the cell population was lower than in metaphase; presumably a number of this class of polyploids fail to divide also.

Aneuploidy was a common feature, and was considered to be related to the mitotic abnormalities frequently seen.

I wish to thank Professor T. F. Hewer for reading this paper, Mr. Clifford Jeal for technical assistance, Mr. Dennis White for photography, and the British Empire Cancer Campaign for financial support.

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PROTEIN-BOUND AZO DYES IN THE SERUM OF RATS FED 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE

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SEVERAL aminoazo dyes are known to induce liver tumours when fed to rats (Miller and Miller, 1953, 1955). The dyes which appear in the liver after feeding have been classified as "free", "polar bound" and "non-polar bound" by Miller and Miller (1947). The free dye is that fraction which can be extracted by ethyl alcohol from the dried tissue protein. The bound dye, in contrast, cannot be removed from the intact proteins by even the most rigorous extraction, but is only liberated after alkaline hydrolysis. The non-polar fraction of the bound dye can be extracted from the hydrolysis mixture by petroleum ether, whereas the polar fraction, which constitutes the major part, can be extracted only by an ethyl alcohol-ethyl ether mixture. The non-polar dyes have been identified as N-demethylation products, while the polar bound dyes are believed to owe their polar properties to their attachment to some amino acid or peptide fragment released from the liver protein by the alkaline hydrolysis (Miller and Miller, 1952, 1953, 1955; Terayama, Ishidate and Hanaki, 1959).

In rats fed aminoazo dyes, either continuously at a low level, or in a single large dose, the only organ in which bound dye is found is the liver (Miller and Miller, 1947; Gelboin, Miller and Miller, 1958). Studies on the accumulation of protein-bound dye in the liver under various conditions indicate that the formation of this bound dye has a role in the carcinogenic process, possibly through deletion of the protein involved in the binding (Miller and Miller, 1947, 1953, 1955; Miller *et al.*, 1949). However, the observation that high levels of bound dyes are found in the livers of rats fed the weakly carcinogenic 2-methyl-4-dimethylaminoazobenzene and 3-methyl-4-dimethylaminoazobenzene (Miller *et al.*, 1949) and in the livers of refractory rats after feeding 3′-methyl-4-dimethylaminoazobenzene (Griffin *et al.*, 1955; Ward and Spain, 1957; Spain and Clayton, 1958) suggests that the formation of bound dye in the liver may be a necessary, but not sufficient, requirement for the induction of liver tumours by aminoazo dyes (Miller and Miller, 1955).

Besides its presence in the liver, a relatively small amount of bound dye has also been detected in the blood plasma of rats fed 4-dimethylaminoazobenzene for two months (Miller and Miller, 1947). No further attention has, however, been given to the occurrence or possible importance of bound dye in blood or serum. We have, therefore, undertaken a study of this aspect of the problem. The present work reports the determination of bound dye in the serum and observations on the protein fractions to which it is attached.

MATERIAL AND METHODS

Reagents.—3′-Methyl-4-dimethylaminoazobenzene (3′-MeDAB; m.p. 120·4–121·2° C.) was prepared by coupling diazotized *m*-toluidine with dimethylaniline as described by Miller and Miller (1948).

The borate-phosphate buffer used in the electrophoresis experiments had an ionic strength of 0·05 and contained 2·34 g. $NaH_2PO_4 . 2H_2O$ and 4·29 g. $Na_2B_4O_7 . 10 H_2O$ per litre. The pH was 8·45 at 22° C.

Treatment of animals.—Male albino rats (weight 180–220 g.) of the Wistar strain were provided by the National Nutrition Research Institute, Pretoria. The animals were housed in wire cages at 20°C. and fed *ad libitum* on the Institute's stock diet (protein, 20 per cent; ash, 7·4 per cent; main component: maize meal, 56 per cent) and tap water.

A single dose of 50 mg. of 3′-MeDAB in 2 ml. of olive oil was administered by stomach tube to rats which were fasted for four hours before dosing, and their blood was drawn 45 hours later. Preliminary tests had shown that the level of bound dye in the serum and the liver reached a maximum at about 40 hours after dosing.

For the collection of serum, the animals were anaesthetized with ether, the abdominal cavities exposed and the blood was obtained from the abdominal aorta. The blood was left to clot, centrifuged and the serum obtained.

Column electrophoresis.—Column electrophoresis of the serum was performed as described by Gedin and Porath (1957) in a vertical column (diameter 1·75 cm., length 150 cm.) filled with Munktell's cellulose powder. The column was cooled with water of 4° C. in the outer jacket. The serum was dialysed against two changes of buffer (1 litre). Eight ml. of dialysed serum (i.e. about 500 mg. of protein) was applied to the column. Using a voltage of 1500 V and a current of 25 mA the electrophoresis was carried out for 52 hours. After electrophoresis, the separated proteins were displaced from the column with buffer and the effluent was collected in fractions of 3·8 ml. The protein content of each fraction was determined by measuring the absorption at 280 mμ. The optical densities were plotted against the numbers of the fractions and the resulting pattern was used as a guide for combining the fractions to obtain five serum protein fractions, namely, pre-albumin, albumin, α-globulin, β-globulin and γ-globulin. These combined fractions were dialysed free from salts against distilled water, freeze-dried and then dried *in vacuo* over P_2O_5. The identity of these fractions was ascertained by paper electrophoresis on horizontal strips of Whatman 3 MM filter paper.

Estimation of dye.—For the determination of free, polar bound and non-polar bound dye in the serum and in the serum protein fractions, a modification of the methods of Miller and Miller (1947) and of Ward and Spain (1957) was used.

The free dye was extracted from salt-free, lyophilized protein samples (50–80 mg.) with 50 ml. of absolute ethyl alcohol for 15 minutes at 75° C. After centrifugation the residue was re-extracted twice with a boiling mixture of 30 ml. of absolute alcohol and 10 ml. of peroxide-free diethyl ether. Determination of free dye in each subsequent extract proved that this procedure gave quantitative extraction of the free dye. The extracts were combined, concentrated to 20 ml., chilled, 25 ml. of chilled 7 N HCl added, and the optical absorption measured after 15 minutes at 505 mμ, using a 10 cm. absorption cell. The blank correction was

obtained after reduction of the azo dye by addition of 0·45 ml. of a saturated solution of stannous chloride in concentrated hydrochloric acid.

After these extractions the proteins were dried *in vacuo* over P_2O_5. A weighed amount of the dried protein was hydrolysed in an aqueous KOH-alcohol mixture and the non-polar and polar bound dyes were determined according to the method of Miller and Miller (1947). Blank corrections were again obtained after reduction with stannous chloride.

The results of the measurements have been calculated as mμmoles of azo dye per 100 mg. of protein, as described by Ward and Spain (1957).

Control experiments in which 0·3 mg. of 3′-MeDAB was added to 5 ml. of normal rat serum and allowed to stand overnight at 4° C. before extraction, showed that no *in vitro* formation of bound dye either in the polar or non-polar form occurred under the above conditions.

Spectrum of bound dye.—The absorption spectra of polar bound dye obtained from the liver (according to the method of Ward and Spain, 1957) as well as from the serum of the same rats were obtained as the difference between the spectra in ethyl-alcohol-7 *N* hydrochloric acid (4 : 5) before and after reduction with stannous chloride.

RESULTS

Free electrophoresis of serum obtained 45 hours after administration of 50 mg. of 3′-MeDAB showed that the pattern of the serum proteins was completely normal.

The amounts of free and non-polar and polar bound dye in two sera, each obtained from three rats 45 hours after administration of 50 mg. of 3′-MeDAB, are shown in Table I. From this it is seen that in addition to the free dye, appreciable amounts of polar bound dye were present. The non-polar bound dye concentration, however, was small.

TABLE I.—*Azo Dye Concentrations in Sera of Rats after Administration of 50 mg. of 3′-MeDAB. Concentrations are Expressed as mμmoles Dye per 100 mg. Protein*

	Serum A	Serum B
Free dye	81	95
Non-polar bound dye	2	2
Polar bound dye	20	36

The elution curves obtained after column electrophoresis of two sera, A and C, each from three rats treated with 50 mg. of 3′-MeDAB showed a significant pre-albumin component. According to Garbers and Joubert (1958) this effect is due to an increased electrophoretic mobility of α_1-glycoprotein in the complexing borate buffer used. From analysis of the elution curves it was estimated that the pre-albumin fractions used for aminoazo dye determinations contained up to 50 per cent of albumin. The relative proportions of the protein fractions (Table II) were obtained from the areas of the corresponding portions of the elution curves.

The amounts of dye associated with the serum protein fractions are also given in Table II. The quantities of α-globulin available for the dye determinations were small, and consequently the values calculated for the amount of dye in this protein fraction are less reliable.

TABLE II.—*Azo Dye in Protein Fractions of Rat Serum After Administration of 50 mg. of 3′-MeDAB*

Serum	Serum protein fraction	Serum protein composition (per cent of total serum protein)	Concentration of dye in fraction (mμmoles/100 mg. protein)			mμmoles polar bound dye in protein fractions of 100 mg. total serum protein (calculated)
			Free dye	Non-polar bound dye	Polar bound dye	
A	Pre-Albumin	17	5	1	14	2·4
	Albumin	37	101	2	30	11·7
	α-globulin	7	(4)	0	(3)	0·2
	β-globulin	20	0	0	3	0·6
	γ-globulin	18	7	0	3	0·5
C	Pre-Albumin	16	0	1	11	1·8
	Albumin	33	12	1	22	7·3
	α-globulin	11	0	1	(4)	0·4
	β-globulin	20	0	0	2	0·4
	γ-globulin	20	0	0	3	0·6

The free dye in the serum is associated essentially with the albumin. However, only insignificant amounts of free dye could be detected in the pre-albumin fractions, although a large part of these fractions is albumin. It therefore appears that the free dye is adsorbed on the slower moving albumin.

Relatively large amounts of polar bound dye are associated with the albumin, and only very small amounts of polar dye seem to be bound to α-globulin, β-globulin and γ-globulin. The polar dye found in the pre-albumin fraction may be accounted for as albumin-bound dye. Calculation of the total amount of polar dye bound to each protein fraction per 100 mg. of total serum proteins shows that approximately 90 per cent of the polar dye in the serum is bound to albumin (Table II, last column).

In order to compare the protein-bound dyes found in the serum to those found in the liver, the absorption spectra of the dyes released in either case, by alkaline hydrolysis, have been measured in acid-ethyl alcohol solution. These spectra were of similar shape in the wavelength range 400 mμ to 600 mμ, but the maximum occurred at 520 to 522 mμ in the case of dye released from serum or serum albumin and at 525 mμ in the case of that from liver protein.

DISCUSSION

It is known that most of the free (demethylated) aminoazo dyes in the blood are associated with the erythrocytes (Miller, Miller and Baumann, 1945). The demonstration of free dye in the serum of rats fed a single dose of 50 mg. 3′-MeDAB is, therefore, of interest because similar quantities of free 3′-methyl-4-aminoazobenzene have been found in the blood of rats fed 3′-MeDAB in the diet for 15–33 days (Miller *et al.*, 1958).

Of greater importance is the present demonstration of substantial amounts of polar bound dye in the serum. The dye released from serum proteins by alkaline hydrolysis is similar in solubility properties (e.g. in petroleum-ether and ethanol-ether mixtures) to that bound to liver proteins in the same rats. The absorption spectra were also generally similar, except that a small difference in wavelength of maximum absorption was observed. The significance of this difference is

uncertain. Terayama *et al.* (1958) and Terayama, Kusama and Aoki (1958) fractionated protein-bound DAB derivations after protein hydrolysis into a fraction with λ_{max} 514 mμ and another with λ_{max} 522 mμ and they considered that the first fraction contained a primary amino group, and the second a secondary amino group. The question whether similar differences exist between the dyes bound to serum and liver proteins cannot be answered at this stage.

Unpublished work in this laboratory has shown that the amount of bound dye per 100 mg. of protein in the serum is approximately half that in the liver of the same rats at all times between 12 and 45 hours after feeding. Taking into account the relative weights of liver and serum proteins in the rat, the total quantity of bound dye in the serum is approximately one-quarter of that in the liver. This constitutes evidence that the appearance of bound dye in the serum parallels that in the liver. From calculations based on a half-life of $3\frac{1}{2}$ days for the liver protein-bound dye (Miller and Miller, 1952; Gelboin *et al.*, 1958) it also appears unlikely that the considerable amount of bound dye in the serum has been formed from the liver protein-dye complex. It should furthermore be noted that the bound dye in the serum is associated with the electrophoretically fast moving albumin fraction, while in the liver the bulk of the bound azo dye has been found in a slow moving fraction of the soluble proteins (Sorof *et al.*, 1951).

The demonstration that the bound dye in the serum is associated with the albumin fraction is of interest in view of the important role of the microsomes of liver cells in the process of albumin synthesis (Peters, 1957, 1959; Campbell, Greengard and Kernot, 1960) as well as in the process of incorporation of aminoazo dyes into proteins during their synthesis (Hultin, 1956, 1957; Gelboin *et al.*, 1958).

These considerations suggest that the binding of aminoazo dyes is not a highly specific process, involving a single protein, but rather that it tends to occur on a number of proteins (see also Terayama and Otsuka, 1960).

The relative importance of incorporation of various aminoazo dyes into liver and serum proteins and the mechanism of binding is now being studied in this laboratory.

SUMMARY

Significant quantities of free and bound dye have been observed in the serum of rats fed a single dose of 3′-methyl-4-dimethylaminoazobenzene. After column electrophoresis on cellulose, these dyes were shown to be associated with the albumin fraction. The results do not support the contention that binding of aminoazo dye to protein in the liver is highly specific.

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SURVEY OF PAPERS

PHILLIPS finds that cancer mortality in females has shown a significant decrease in Canada through the years 1941 to 1958 while, in males, there has been a significant increase. Mortality trends for the major sites of cancer have also been analyzed for significant changes (p. 1).

CAMPBELL finds that for 50 years the joint mortality rate from tuberculosis and for all types of cancer has remained remarkably constant for the population of Australia. He discusses the significance of this relationship, previously shown for some other countries (p. 10).

HENDERSON and CURWEN describe a study of the histological type of tumour in nearly 40,000 lung cancer patients from the South-West of England. They find that the distribution of the main histological types varies considerably according to sex and method of diagnosis, and that the proportion of oat-cell tumours decreases with increasing age (p. 19).

MUIR gives an account of the incidence and morbid anatomy of malignant tumours of the liver, pancreas and peritoneum in Singapore, and compares the prevalence of these cancers with that in some other countries (p. 30).

STANSFIELD finds no abnormalities of the blood or bone marrow of African children with malignant lymphoma which would be considered " leukaemic " (p. 41).

SICHER and WATERHOUSE have assessed thyroid function, determined by serial uptake and excretion measurements of the isotope ^{131}I, in relation to age, stage and serum cholesterol, in a series of cases of breast cancer localised to the breast or regional glands (p. 45).

KREYBERG has compared British and Norwegian studies of lung cancer in relation to smoking. The similarity of the findings leads to the conclusion that tobacco—especially cigarette-smoking—is the essential causative factor involved in the development of epidermoid carcinomas and oat-cell carcinomas of the lung (p. 51).

DE finds, from a study of twenty cases of carcinoma of the cervix, that there is correlation between response to irradiation and average chromosome number. Tumours showing polyploidy (more than sixty chromosomes) are usually radioresistant, and conversely (p. 54).

VAN DEN BRENK has studied the effect of breathing high pressure oxygen on the radiosensitivity of Ehrlich's tumour (solid form) in mice. He finds that this leads to a significant increase in radiocurability (assessed by cure of tumours 8 weeks after irradiation) with a dosage reduction by a factor of 2·4 (p. 61).

DICKENS and JONES have made a study of the effect of subcutaneous injection into rats of a number of chemically and pharmacologically active lactones and related substances. Among the substances shown by them to produce tumours, in addition to β-propiolactone which is already known to be carcinogenic, are the antibiotics patulin and penicillic acid (p. 85).

HUGHES reports studies in which the cultivated mushroom was used as an experimental organism. Neoplasms were only induced by treatment with Diesel-oil soot at an early stage in differentiation. Cytological, histochemical (RNA) and growth rate studies were made to

determine the nature of the change involved in neoplasm formation and which cells gave rise to the neoplasms. The evidence suggests that the susceptible cells are metabolically active intermitotic cells (p. 101).

Bather describes a monocytic leukaemia in mice which results from infection by a virus originally isolated from S37 sarcoma tissue and from spleens of animals surviving S37 sarcoma growth and regression (p. 114).

Wakonig-Vaartaja has made chromosome analyses on leukaemias induced in mice by S37 and Friend viruses. The occurrence of diploid cases shows that aneuploidy is not the primary cause of neoplasia in these leukaemias (p. 120).

Baldwin, Cunningham and Pratt find that squamous cell carcinomas often result from skin-painting of mice with a commercial motor engine oil additive preparation. The additive studied is irritant for mouse skin, causing inflammatory changes and often ulceration (p. 123).

Pullinger finds that continued high level exposure to oestrone after breeding and ovariectomy brings the incidence of mammary tumours from the low level found after ovariectomy only to a level (26 per cent developing carcinoma) which is the normal spontaneous incidence for breeders of the strain of mice used (p. 127).

Marchant finds that no breast tumours develop in male IF mice treated with methylcholanthrene and subsequently castrated and grafted subcutaneously with an ovary, although some proliferation of breast tissue occurs. Animals castrated before carcinogen treatment do not develop breast tumours either (p. 133).

Emmelot, Hampe, Bos and Reyers have determined the deoxycytidylate deaminase and thymine catabolism in 105,000 $\times$ g supernatants from liver tissue and hepatomas of the mouse and rat. They find no correlation between the activity of the former enzyme and the histology of the transplanted rat hepatomas, and cannot consider the enzyme as a proper parameter of the cell origin of these tumours (p. 138).

Doxey has measured the sulphydryl content of HeLa cells and chick fibrocytes (p. 146).

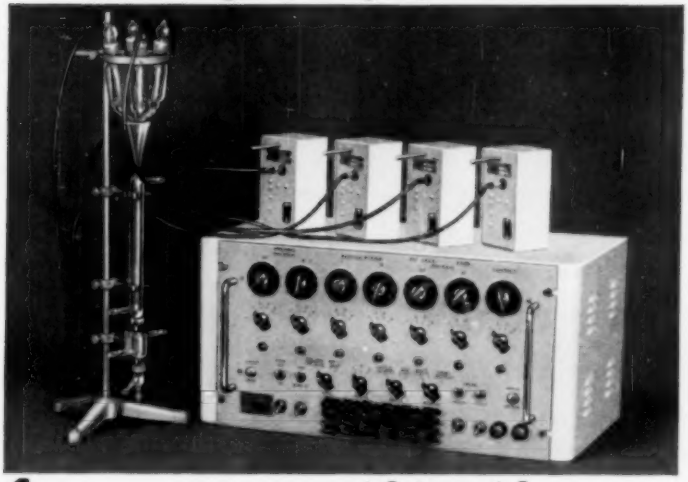
Calcutt, Doxey and Coates have studied the effects of a series of different chemical carcinogens on various susceptible and non-susceptible animal tissues. They suggest, from a consideration of their findings, that an elevation of tissue —SH levels is an essential prerequisite for tumour formation (p. 149).

Calcutt finds that thiamine hydrochloride or calcium pantothenate by intraperitoneal injection causes a short-lived decrease in liver —SH values in mice (p. 157).

Meek reports studies on the distribution of cellular DNA values in mouse ascites tumour cells in interphase and mitosis. Whilst an increase in DNA content above the normal cell level is a common feature, it is concluded that those cells with unusually high values show a greatly reduced rate of proliferation (p. 162).

Dijkstra and Joubert find significant quantities of free and bound dye, associated with the albumin fraction, in the serum of rats fed a single dose of 3′-methyl-4-dimethylaminoazobenzene, suggesting that binding of aminoazo dye to protein in the liver is not highly specific (p. 168).

Automatic Biological Assay Apparatus



for quantitative measurement of the activity of drugs

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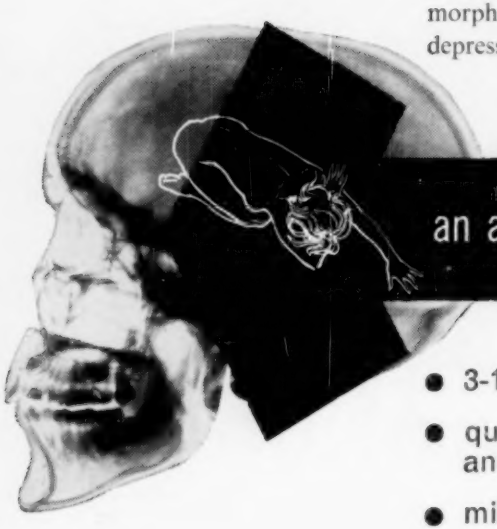

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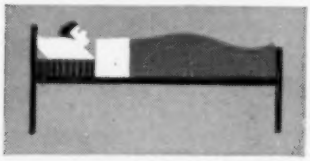

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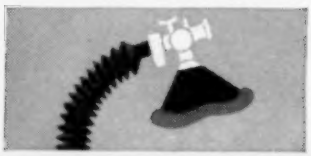

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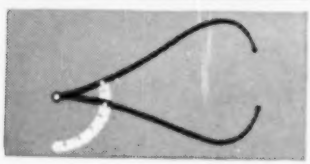

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